A COMPREHENSIVE ANALYSIS OF THE MICROBIAL DIVERSITY ASSOCIATED WITH *EUNICEA FUSCA* AND RELATED *PLEXAURIDAE* OCTOCORALS, AND THE ISOLATION OF NEW NATURAL PRODUCTS FROM SELECTED, ASSOCIATED BACTERIA

BY

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DOCTOR OF PHILOSOPHY

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Dr. Russell Kerr (Supervisor)

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ABSTRACT

Marine invertebrates, including octocorals, are a prolific source of marine natural products (MNPs) and are also known to host diverse microbial communities. It is well-established that marine microbes are producers of bioactive secondary metabolites. Accordingly, it is hypothesized that bioactive metabolites extracted from some marine invertebrates may actually be produced by associated microbes rather than the invertebrate host.

This research aimed to characterize the microbial community of *E. fusca* and related *Plexauridae* octocorals and to discover novel, bioactive compounds biosynthesized by associated microorganisms. *E. fusca* was the main focus of this study because its microbial community has not been thoroughly characterized, and it is the sole source of the potent anti-inflammatory diterpenes fuscol, eunicol, and the fuscosides. Investigations of the unexplored *E. fusca* microbial community could therefore lead to the discovery of novel MNPs from unexplored microbes, as well as provide insights into the biosynthetic source of these diterpenes.

The microbiome of Floridian and Bahamian *E. fusca* and related *Plexauridae* octocorals collected in June 2009 were characterized using culture-independent (454-pyrosequencing, denaturing gradient gel electrophoresis, cloning, and species-specific primers) and -dependent (dilution plating) methods. The culture-independent analysis revealed that *E. fusca* hosted a geographically-heterogeneous bacterial community, where *Endozoicomonas* relatives dominated the Florida sites, and *Mycoplasma* relatives dominated at The Bahamas sites. There were, however, a few stable bacterial associates found in all *E. fusca* at all sites, including relatives of the *Endozoicomonas*, *Mycoplasma*, and *Oceanospirillales*, suggesting that these bacteria may be critical to holobiont functioning, and differences in environmental conditions at the locations may affect the dominant community member. *E. fusca*'s bacterial community also differed from that of closely-related *Plexauridae* octocorals and the surrounding seawater. All octocorals contained the same clade of dinoflagellate, *Symbiodinium* B1/B184. However, the fungal and archaeal communities were not consistent between *Plexauridae* species or location.

In the culture-dependent study, 137 unique bacteria and 11 unique fungi were cultured from the *Plexauridae* octocorals; 31 of the isolated bacteria were putative novel species based on 16S rDNA analysis (<97% sequence identity of 16S rDNA). Two of the novel species, *Endozoicomonas euniceicola* sp. nov. and *Endozoicomonas gorgoniicola* sp. nov., had nearly identical 16S rDNA sequences to the dominant members in the culture-independent bacterial community, providing the extraordinary opportunity to explore the biology and chemistry of these putative octocoral symbionts. These two bacteria were formally characterized, revealing that they were genotypically, phenotypically, and chemotypically different from the three *Endozoicomonas* spp. cultures previously isolated from other marine invertebrates.

From the culture-dependent library, selected bacteria were fermented and analyzed for MNP production. Under the evaluated fermentation conditions, two bacteria were found to produce novel MNPs. A *Labrenzia* sp. produced a family of novel fatty acid derivatives, and a *Euzebyella* sp. produced a novel 2,5-dialkylresorcinol with good gram-positive bioactivity.

In conclusion, *Eunicea fusca* hosts a geographically heterogeneous and diverse microbial community that differs from that of other closely-related *Plexauridae* octocorals and the surrounding environment. This microbial community has a varied metabolic repertoire, which can ultimately lead to the discovery of novel, microbial MNPs.

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I would like to thank my Supervisor, Dr. Russ Kerr for giving me the opportunity to carry out my PhD in his MNP laboratory, his guidance and support the past 4.5 years, and his continual support in my future endeavors. Russ's advice that "You only do your PhD once" made me truly enjoy and appreciate every moment (even the challenging ones!) of my PhD.

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LIST OF ABBREVIATIONS

ABC = adenosine triphosphate (ATP)-binding cassette

ACN = acetonitrile

antiSMASH = Antibiotics and Secondary Metabolite Analysis SHell

ATCC = American Type Culture Collection

avg. = average

BFM3 = Bacteria Fermentation Media 3

BLAST = Basic Local Alignment Search Tool

BOX PCR = BOX-A1R-based repetitive extragenic palindromic-PCR

bp = base pair

BS = The Bahamas

bTEFAP = bacterial Tag-Encoded FLX Amplicon Pyrosequencing

^{13}C = carbon NMR

CD_3OD = deuterated methanol

CECT = Colección Espagñola de Cultivos Tipo

CNI = Close-Neighbor-Interchange

COG = Clusters of Orthologous Groups of protein

COSY = correlation spectroscopy

d = doublet (^1H NMR)

DCM = dichloromethane

dd = doublet of doublets (^1H NMR)

ddd = doublet of doublets of doublets (^1H NMR)

DGGE = denaturing gradient gel electrophoresis

diH_2O = distilled, deionized water

DMSO = dimethyl sulfoxide

DMSP = dimethylsulfoniopropionate

DNA = deoxyribonucleic acid

DSMZ (or DSM) = Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

dt = doublet of triplets (^{1}H NMR)

E = Expectation

E' = Shannon equitability index

EDTA = ethylenediaminetetraacetic acid

EF = *Eunicea fusca*

ELSD = evaporative-light scattering detector

ENA = European Nucleotide Archive

ES = *Eunicea* sp.

ESI = electrospray ionization

EtOAc = ethyl acetate

EtOH = ethanol

eV = electron volts

FAME = fatty acid methyl ester

FDA = Food and Drug Administration (USA)

Fe = iron

FISH = fluorescence *in situ* hybridization

FL = Florida

FPPS = farnesyldiphosphate synthase

G+C mol% = guanine and cytosine molecular percentage

G-3-P = glycerol-3-phosphate

GA = Gibberellic Acid

GBR = Great Barrier Reef

GC = Gas Chromatography

gDNA = genomic deoxyribonucleic acid

GI = genomic island

^{1}H = proton NMR

H' = Shannon diversity index

HMBC = heteronuclear multiple bond correlation

HPLC = high performance liquid chromatography

HRMS-MS = tandem high resolution mass spectrometry-MS

HSQC = heteronuclear single quantum correlation

HTG = horizontally transferred gene

HTS = high-throughput sequencing

IC_{50} = half maximal inhibitory concentration

ID = identity

ITS = internal transcribed spacer

J in Hz = J-coupling constant in Hertz

kb = kilobase pair

KCTC = Korean Collection for Type Cultures

KEGG = Kyoto Encyclopedia of Genes and Genomes

LC-HRMS = liquid chromatography coupled to HRMS

LMG = Laboratorium voor Microbiologie, Universiteit Gent, Belgium

m = multiplet (^{1}H NMR)

m/z = mass to charge ration

MA = marine agar

MALDI-TOF MS = matrix-assisted laser desorption/ionization–time of flight MS

marC = multiple antibiotic resistance C

MB = marine broth

Mb = megabase pair

ME = Minimum Evolution

UPGMA = unweighted pair group method with arithmetic mean

MeJA = methyl jasmonate

MeOH = methanol

MHz = megahertz

MIC = minimum inhibitory concentration

mMB = modified marine broth

MNP = marine natural product

MRSA = methicillin- resistant *Staphylococcus aureus*

mTSB = modified Trypticase Soy Broth

NADH = Nicotinamide adenine dinucleotide

NB = nutrient broth

NBRC = NITE (National Institute of Technology and Evaluation) Biological Resource Center, Japan

NCBI = National Center for Biotechnology Information

NCCB = Netherlands Culture Collection of Bacteria

NGS = next generation sequencing

NJ = Neighbor-Joining

nMDS = nonmetric multidimensional scaling

NMR = nuclear magnetic resonance

No. = number

NP = natural product

NR = non-redundant protein sequences (GenBank database)

NRP = non-ribosomal peptide

NRPS = non-ribosomal peptides synthetase

OD = optical density

ORF = open reading frame

OTU = operational taxonomic unit

PCA = principal component analysis

PCR = polymerase chain reaction

PDA = photodiode array

PK = polyketide

PKS = polyketide synthase

PS1 = *Plexaura* sp. 1

PS2 = *Plexaura* sp. 2

QS = quorum sensing

rDNA = ribosomal deoxyribonucleic acid

RDP = Ribosomal Database Project

REF = reference

RNase = ribonuclease

ROV = remotely operated vehicle

RP-HPLC = reverse phase HPLC

RT = retention time

RTL = Research and Testing Laboratories

s = singlet (^{1}H NMR)

SA = salicin

SCUBA = self-contained underwater breathing apparatus

SD = synthetic derivative

SDS = sodium dodecyl sulfate

SFSW = sterile filtered seawater

SRA = Short Read Archive

SRR = sequence read run

SW = seawater

SYMB = peptone maltose yeast broth

t = triplet (^{1}H NMR)

T1SS = type I secretion system

TCA cycle = tricarboxylic acid cycle

TE = Tris-EDTA

TEM = transmission electron microscopy

TLC = thin layer chromatography

TRAP = tripartite ATP-independent periplasmic

TrEMBL = Translated of European Molecular Biology Laboratory Nucleotide Sequence Data Library

T-RFLP = terminal-restriction fragment length polymorphism

UV = ultraviolet

V = 16S rDNA hypervariable region (Chapter 2)

v = volts (Chapter 3)

v/v = volume per volume

VRE = vancomycin-resistant *Enterococci*

VREF = vancomycin-resistant *Enterococci faecium*

w/v = weight per volume

g = gravity

YM = yeast-malt medium

δ = NMR chemical shifts

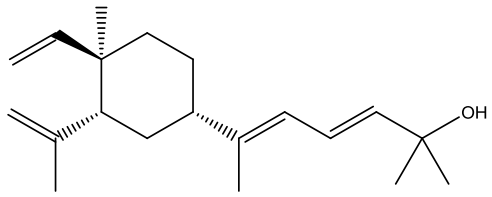
CHAPTER 1: AN INTRODUCTION TO MARINE NATURAL PRODUCTS AND OBJECTIVES OF THIS PHD THESIS

1.1 Introduction to Marine Natural Products

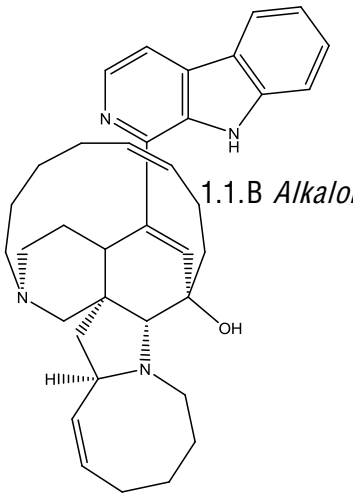
1.1.1 Definition and Classification of Marine Natural Products

Marine natural products (MNPs) are secondary metabolites isolated from marine organisms that are not necessary for the basic sustenance of life, but confer some adaptive advantage to the organism.[1] For example, in their natural environment coral MNPs may serve as fish feeding deterrents[2] or antifouling agents.[3] These bioactive compounds, in turn, can be used by humans as pharmaceutical agents or in personal health care products.

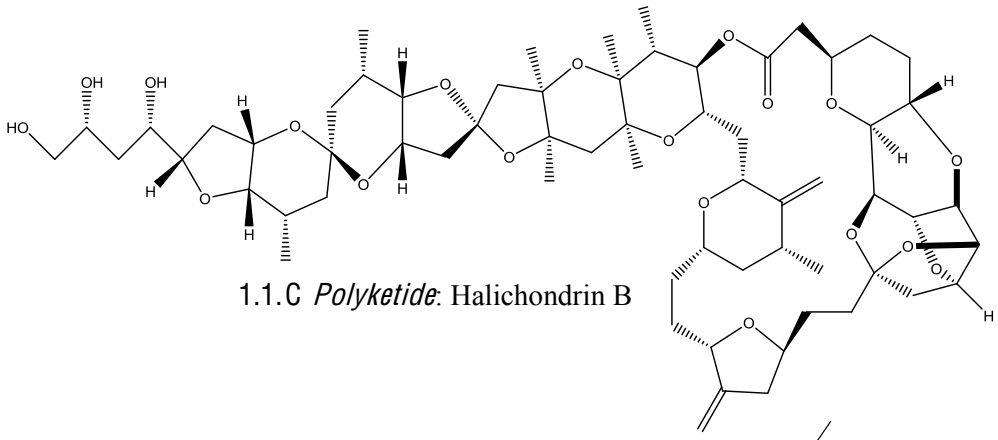
MNPs, and natural products (NPs) in general, fall into one of the following families of secondary metabolites. (1) Terpenes are derived from isoprene units (C_5H_8) where the isoprene units are linked together via head-to-tail condensation reactions carried out by terpene synthases to form chains or rings (*e.g.* fuscol, Figure 1.1.A). (2) Alkaloids are nitrogen-containing, cyclic and basic compounds derived from one of five amino acids (lysine, ornithine, tryptophan, phenylalanine, or tyrosine) or acetate units (*e.g.* manzamine A, Figure 1.1.B). (3) Polyketides (PKs) are NPs biosynthesized by polyketide synthases (PKSs) most commonly composed of repeating acetate units produced as an evolutionary off-shoot of fatty acid metabolism (*e.g.* halichondrin B, Figure 1.1.C). (4) Non-ribosomal peptides (NRPs) are NPs composed of amino acid building blocks joined via amide linkages assembled via non-ribosomal peptide synthatases (NRPSs) in a similar manner to PKs (*e.g.* kahalide F, Figure 1.1.D). (5) Shikimic acid derivatives are compounds derived from shikimic acid and are precursors to flavonoids (*e.g.* 2,5-dihydrophenylalanine, Figure 1.1.E) and the aromatic amino acids, phenylalanine and tyrosine. (6) Miscellaneous NPs, including peptides (*e.g.* ziconotide, Figure 1.1.F) and nucleosides (*e.g.* cytarabine, Figure 1.1.G), are typically biosynthesized via "primary" metabolite biosynthetic pathways.



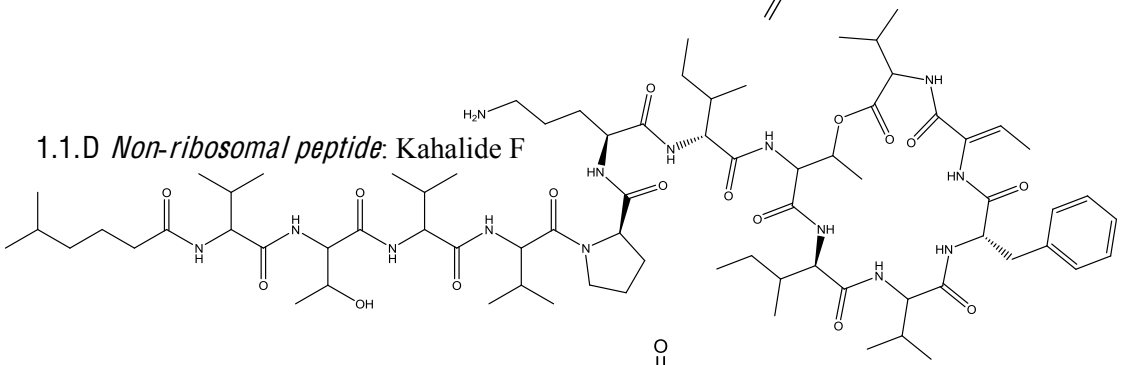
1.1.A *Terpene*: Fuscol



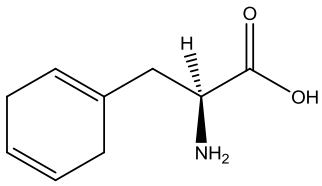
1.1.B *Alkaloid*: Manzamine A



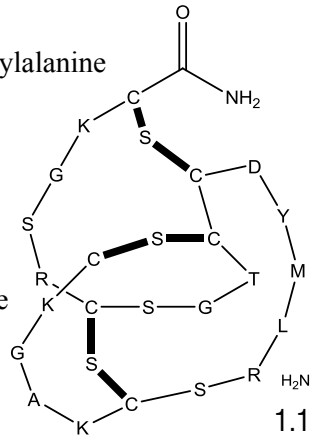
1.1.C *Polyketide*: Halichondrin B



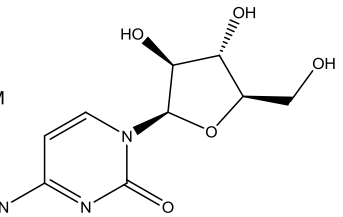
1.1.D *Non-ribosomal peptide*: Kahalide F



1.1.E *Shikimic acid*: 2,5-dihydrophenylalanine



1.1.F *Peptide*: Ziconotide



1.1.G *Nucleoside*: Cytarabine

Figure 1.1 Examples of natural products from different families of secondary metabolites.

1.1.2 Why do Natural Products Serve as Superior Drugs?

Natural products make up a significant portion of agents currently used as drugs. In fact, 64% of all drugs, and 69% of anti-bacterial, -fungal, and –parasitic drugs, are NP-derived or –inspired.[4] The reason why NPs are of great interest in drug development is because of their structural complexity, molecular diversity, high selectivity, and specific biological activities based on unique mechanisms of action.[4,5] Through evolution, all NPs have been biologically "validated" to serve a particular biological function, and thus most, if not all, NPs have some receptor-binding activity, although finding the natural receptor can be challenging.[6] The terrestrial environment has been the focus of drug discovery for centuries, and it has provided many potent drugs, such as the anticancer drug, Taxol®, derived from the leaves and bark of various *Taxus* species[7,8] and endophytic fungi.[9,10] However, the marine environment holds much potential as a source of novel drugs, as in-depth exploration of this environment only began in the 1970s.[11,12] Thus, the tides are turning in favor of exploring the untapped marine environment for novel drug candidates.[13]

1.1.3 Why Search the Marine Environment for Natural Products?

In addition to its limited exploration, our oceans are an attractive environment to explore for novel bioactive agents due to their tremendous biodiversity. The oceans occupy more than 70% of the earth's surface and contain an estimated 1-2.2 million marine species.[14,15] Most marine organisms inhabit demanding marine environments, so it is hypothesized that they possess diverse and structurally-interesting secondary metabolites in order to aid in their survival. In addition, the high potency of MNPs (due to their release in an aqueous environment[16]) makes them highly desirable targets of drug discovery programs.

1.1.4 Marine Natural Product History, Success Stories, and the Supply Issue

The field of MNPs is a relatively young one, beginning in the 1950s[17] with the investigation of easy to collect marine invertebrates inhabiting shallow waters. In-depth investigations of the marine environment, however, really only began in the 1970s with the

development of reliable SCUBA diving equipment, followed by the introduction of remotely operated vehicles (ROVs) for marine scientific research.[4] As of 2012, more than 22,000 novel MNPs had been isolated and characterized,[18] many of which belonged to novel chemical classes only found in the marine environment, and many of which had extraordinary biological activities.[4] Of the 22,000 MNPs isolated, seven have gained FDA approval as marketed drugs, and of these seven, two are used as the isolated chemical structures, and five are synthetic compounds inspired from a MNP.[4] Table 1.1 lists the seven MNPs currently in the market, and Figure 1.1.F-G (p. 3) and Figure 1.2.A-E show their structures.

Table 1.1 Descriptions of the seven marine natural products currently in the pharmaceutical market.

MNP Name/ Marketed Name (Fig. no.)	Marine organism source	MNP or synthetic derivative (SD)	Current pharmaceutical applications	Citations
Ziconotide/ Prialt® (1.1.F)	Cone snail, *Conus magus*	MNP	analgesic for chronic pain	19
Ecteinascidin-743/ Yondelis® (1.2.A)	Tunicate, *Ecteinascidia turbinata* (symbiotic bacterium, Candidatus *Endoecteinascidia frumentensis*?)	SD	soft tissue sarcomas, relapsed ovarian cancer	20-24
E7389/ **Halaven™,** Eribulin® (1.2.B)	Sponges, *e.g. Halichondria okadai* (symbiotic bacterium?)	SD	refractory breast cancer	25-28
Brentuximab vendotin/ Adcetris™ (1.2.E)	Cyanobacterium, *Symploca* sp. living within sea hare, *Dollabella auricularia*	SD	large cell lymphoma, Hodgkin's lymphoma	29-32
Lovaza® (1.2.C)	Fish	MNP	reduce serum triglycerides	33
Cytarabine, Ara-C® (1.1.G)	Sponge, *Cryptotethia crypta* (symbiotic bacterium?)	SD	acute myelocytic leukemia	17, 34
Vidarabine, Ara-A® (1.2.D)	Sponge, *Cryptotethia crypta* (symbiotic bacterium?)	SD	antiviral	17, 35

Abbreviations: MNP = marine natural product; SD = synthetic derivative

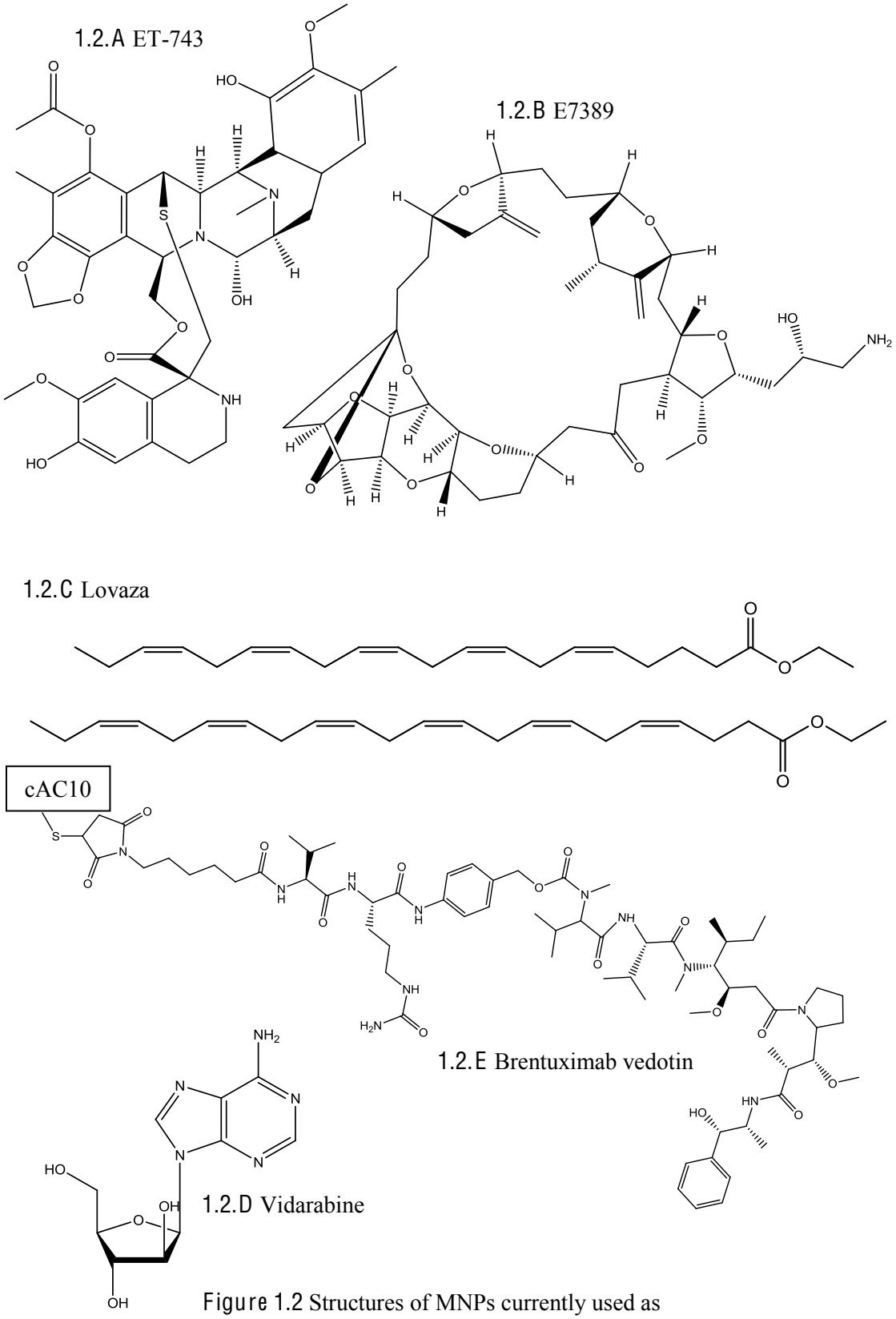


Figure 1.2 Structures of MNPs currently used as pharmaceuticals (also see Figure 1.1.F-G, p. 3).

In addition to these seven MNPs, 13 more are in Phase I/II/ or III clinical trials.[18] Thus, the pipeline of promising MNPs or derivatives is very strong, and many of these agents will likely become drugs in the upcoming years.[36] Furthermore, the current success rate of a MNP (one drug per 3,140 MNPs described) is approximately 1.7 - 3.3 fold better than the industry average of one in 5,000-10,000,[18] suggesting that the marine environment provides an exceptional resource for novel bioactive agents.

Even though MNPs as drugs have been quite successful, the field still faces the obstacle of a shortage of supply of many desired MNPs. The inability to obtain a sufficient supply of a MNP is often the reason why many pharmaceutically-attractive compounds fail to enter clinical trials. In the case of marine invertebrate-derived MNPs, the desired MNP often makes up a small percentage of the overall biomass, and even if it constitutes upwards of 5% of the dry tissue weight of an organism,[37] even the most careful chemical extraction methods are not 100% efficient, resulting in a limited supply of the desired MNPs.

One way to overcome this "supply issue" is to carry out large-scale collections of the source organism. For example, the potent anti-cancer drug, ET-743 (Figure 1.2.A, p. 7), originally isolated from the tunicate *Ecteinascidia turbinata*, only makes up $1x10^{-4}$% of the dry weight of the marine-invertebrate,[38] and the production of 1 g of the highly-demanded drug requires approximately one metric ton (wet weight) of the tunicate.[39] Thus, prior to 1997, the pharmaceutical company PharmaMar collected massive amounts of *E. turbinata* from Caribbean mangroves, and these collections produced sufficient quantities of ET-743 for *in vitro* and preclinical assays.[23]

As one can imagine, large-scale collections of any one organism can cause irreversible damage to the host organism and devastate marine ecosystems, so other methods are desired for a solution to the supply issue. Aquaculture of the marine invertebrate is another possible solution. Referring back to ET-743, in 1997-98, with the progression into phase I trials and a higher demand for ET-743, an aquaculture program for *E. turbinata* was initiated in eleven countries.[23]

In total, the cumulative biomass produced by PharmaMar's aquaculture program was approximately 250 metric tons.[23] Although this program was successful, the final yield after isolation and purification of ET-743 was only about 1 μg g^{-1}. Thus, the cost of aquaculture and the lengthy chemical extraction procedure was not ideal for industrial production. In addition to the high costs incurred from aquaculture, marine eukaryotes are often difficult to culture, and the practice itself can be damaging to the environment through the accidental release of aquaculture wastes or through the introduction of non-native marine invertebrate species to marine ecosystems.

Another potential solution to the supply issue is chemical synthesis or semi-synthesis. This technique has been successful with some of the current MNPs in the market. For example, total chemical synthetic studies were undertaken with halichondrin B (Figure 1.1.C, p. 3) and revealed that the right hand portion of the molecule retained the potency of the parent compound.[27] The synthetic analogue, E7389 (Figure 1.2.B, p. 7), provided adequate supplies for clinical trials, and subsequently, E7389 was approved by the FDA in 2010 for the treatment of refractory metastatic breast cancer.[27] Likewise, total synthesis was attempted with ET-743,[40-46] but low yields (~57%[41]) of the compound rendered chemical synthesis inefficient for manufacturing ET-743 at an industrial scale.[23] As aquaculture and total chemical synthesis were inefficient on the industrial scale, PharmaMar scientists devised a semisynthetic route for production from the bacterium (*Pseudomonas fluorescens*) metabolite cyanosafracin B, which provided enough material for clinical trials.[22] The overall yield of the semi-synthesis was 1.4%, but because the starting material could be obtained in large quantities by bacterial fermentation, this method was favorable and is still used for the industrial production of ET-743.[11]

Although total- and semi-syntheses can be effective, synthesis is often challenging and produces low yields due to the complexity of MNP structures, so other alternatives are desired. Early on, the close resemblance of many marine invertebrate-derived MNPs to terrestrial bacterial metabolites suggested a microbial origin,[11] and research in the past decade has finally provided

solid evidence that some MNPs isolated from marine invertebrates are actually biosynthesized by associated symbiotic microorganisms.[39,47-53] If microbes are the true producers of a desired MNP, then culturing the producing microorganism or identifying the biosynthetic pathway through metagenomics could overcome supply issues through large-scale fermentations or through the expression of identified biosynthetic pathways in a heterologous host. Keeping with the ET-743 story, the dominant symbiont of *E. turbinata* was determined to be Candidatus *Endoecteinascidia frumentensis*, and the putative NRPS gene cluster of ET-743 was identified within the metagenome of *E. turbinata* and likely belongs to this (thus far) uncultivable bacterium.[24] Culturing marine microbes is oftentimes challenging,[54,55] so identifying biosynthetic pathways from metagenomic communities may be the way forward with this solution. The use of microbes as a solution to supply issues will be further discussed in the next section (*1.1.5*), but it should be mentioned that a combination of all of the aforementioned supply issue solutions is necessary to create sustainable supplies of MNPs to enter clinical trials, and ultimately, to become drugs.

1.1.5 Microbial Sources of Novel Marine Natural Products

Even though many of the MNPs currently in the market and in clinical trials were initially isolated from marine invertebrates, it is estimated that ~80% of them are actually biosynthesized by marine microbes.[18] Additionally, other marine invertebrates have been shown to possess symbionts that are responsible for the production of their major bioactive MNP. For example, the bryozoan, *Bugula neritina*, was shown to be heavily dominated by the bacterium Candidatus *Endobugula sertula*, which possessed the bryostatin 1 (anticancer, Alzheimer's MNP) PKS gene cluster,[51] and tunicate, *Lissoclinum patella*, contained the stable bacterial symbiont Candidatus *Endolissoclinum faulkneri*, which was shown to contain the patellazoles (anticancer MNP) PKS pathway.[53] Both bryostatin 1 and the patellazoles were originally isolated from their marine invertebrate hosts in low supply, so having identified these microbial biosynthetic pathways may help to overcome the supply issue of these desired MNPs.

In addition to microbial symbionts providing a sustainable supply of desired MNPs, marine microorganisms also provide a source of *novel*, bioactive compounds. The repeated isolation of known metabolites and reduced hit-rates for macroorganism-derived MNPs has caused researchers to investigate marine microbes in recent years. In the terrestrial environment, it has been shown that microbes are a prolific source of structurally diverse and bioactive metabolites (*e.g.* penicillins, cephalosporins, tetracyclines, cyclosporins, rapamycin, lovastatin, and ivermectins), and the same is believed to be true about marine microbes.[4] Moreover, genomic investigations of isolated microbes have identified silent gene clusters for novel MNPs.[56] Thus, many current MNP researchers are examining marine microbes for their role in the production of known and desired MNPs, as well as for their wealth of novel MNPs.

1.1.6 Octocoral Microbial Communities as a Source of Novel, Microbial Marine Natural Products

One marine microenvironment that remains largely unexplored is octocoral microbial communities. To date, only a handful of microbial diversity studies have been carried out on octocorals.[57-69] From these studies, it has been reported that associated microbes serve as nutrient sources,[70] cycle nutrients in oligotrophic ecosystems,[71] process metabolic wastes,[72] and/or produce secondary metabolites that act as an artificial immune system for the octocorals.[73-75]

Previous studies have shown that octocorals are a prolific source of bioactive compounds.[58,76-79] In particular, the *Plexauridae* family of octocorals is known for its production of interesting secondary metabolites. *Eunicea fusca* is the sole source of the diterpenes, fuscol (Figure 1.1.A, p. 3),[80] the fuscosides,[81] eunicol,[82] and eunicidiol.[83] These compounds and related analogues are potent anti-inflammatory agents with greater potency than the clinically-used drug, indomethacin, in mouse-ear models of inflammation.[84-87] Moreover, their selective inhibitory activity of the 5-lipoxygenase enzyme in the arachidonic acid cascade make them desirable drug targets.[86] However, they cannot progress through clinical trials due to the low yields obtained from current extraction techniques. The closely-related octocoral, *Plexaura homomalla*, is the

source of novel prostaglandins,[88] important mediators in inflammatory diseases, fever, and pain.[39] As previously discussed, the biosynthetic source of these *Plexauridae* MNPs remains elusive. It has been hypothesized that the aforementioned MNPs derived from *E. fusca* are partially or completely biosynthesized by associated microbes.[89,90] Therefore, the isolation of these microbes or the identification of their biosynthetic pathways could provide a consistent yield of the MNPs through the use of large-scale laboratory culture of the microbe or expression of the identified biosynthetic pathways in a heterologous host, respectively. Furthermore, the isolation of novel microbes with new MNP chemistry is anticipated from the untapped and uncharacterized octocoral microenvironments.

1.2 Objectives of Thesis Research

In the Kerr laboratory at the University of Prince Edward Island-Atlantic Veterinary College, there is a large focus on characterizing the microbial communities of Caribbean octocorals and exploring associated microbes for their ability to produce known and novel MNPs. The overall objectives of this thesis research are as follows: (1) Characterize the culture-independent microbial diversity of *Eunicea fusca* and related *Plexauridae* octocorals from geographically-separate locations in Florida and The Bahamas. (2) Identify the culture-dependent microbial diversity of the same octocorals from the same locations. (3) Determine if any of the cultured microbes are novel and significant to the octocorals, and if so, formally characterize these microbes and explore their genetic repetoire. (4) Determine if any cultured microbes produce known diterpene MNPs or novel MNPs with antimicrobial bioactivity.

Figure 1.3 highlights the workflow of this research. First, octocoral samples are collected via SCUBA diving and are processed onsite. The samples then undergo two different studies. (1) In the culture-independent study (Chapter 2), the "total" octocoral microbial community is determined at the time of collection through molecular methods. This is done to determine the major microbial associates that may be involved in the biosynthesis of MNPs or that may be critical to the health of the coral holobiont. (2) In the culture-dependent study (Chapter 3), the

culturable microbial associates are isolated, molecularly characterized, and fermented under a variety of conditions to determine their ability to produce both known and novel MNPs. Bioactivities of novel MNPs are determined through antimicrobial bioassays, and the structures of novel MNPs are elucidated through Nuclear Magnetic Resonance (NMR) Spectroscopy and Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS). In addition, the microbial compositions of the two studies are compared to determine if any of the important, symbiotic microbes from the culture-independent study are cultured. If so, those microbes are further characterized through polyphasic species characterization studies (Chapter 4) and genome sequencing (Chapter 5) to determine their biosynthetic potential and functions within the coral holobiont.

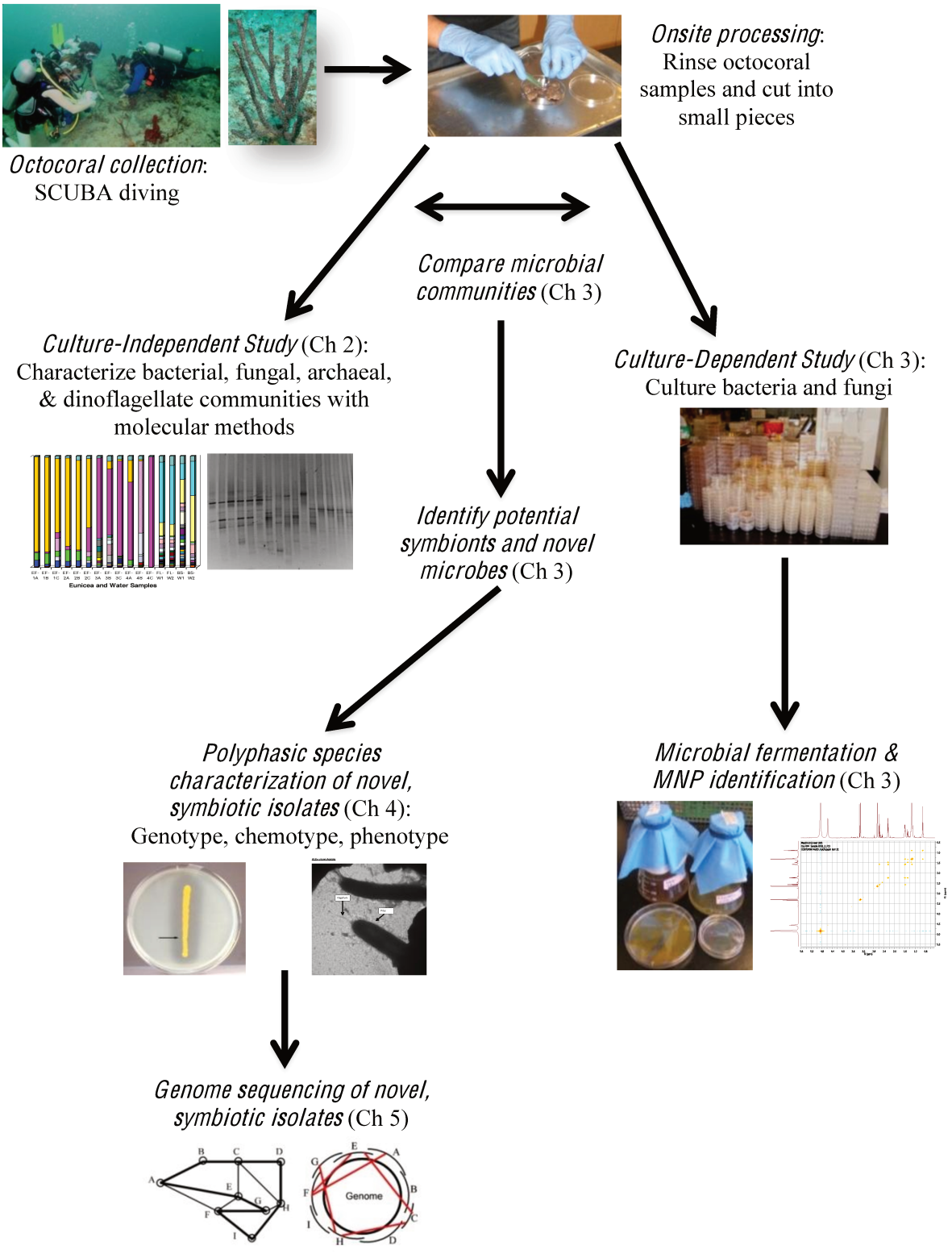


Figure 1.3 Workflow of this MNP research.

1.2.1 Chapter 2: Culture-Independent Study of Eunicea fusca and Related Plexauridae Octocorals to ***Determine "Total"*** *Microbial Composition at the Time of Collection*

In order to determine the "total" microbial communities present in octocorals, culture-independent (metagenomic) studies are required. Denaturing gradient gel electrophoresis (DGGE),[62,64] fluorescence *in situ* hybridization (FISH),[60] clone libraries,[59,63,64,66,67] and most recently, the deep-sequencing technique of 454-pyrosequencing,[65,68,69] are a few of the methods that have been used to determine the microbial composition of octocorals over the past decade. These studies can provide insights into the dominant and stable members of the microbial community that are likely symbionts of the invertebrates. In turn, these symbionts may contribute to the health of the holobiont through the provision of nutrients or protection via biosynthesis of secondary metabolites that protect the invertebrate from invading pathogens[92] and/or act as fish feeding deterrents.[2] This baseline data will determine the healthy, microbial community of these octocorals, and in the future, this data can be used to determine their health status in their natural environment.[93] In the case of *E. fusca*, a dominant and stable member of the microbial community may also provide insights into the biosynthesis of its diterpenes.

Chapter 2 (pp. 270-113) will discuss the culture-independent study of *E. fusca* and related *Plexauridae* octocorals detailing their bacterial, fungal, dinoflagellate, and archaeal communities.

1.2.2 Chapter 3: Culture-Dependent Study of E. fusca and Related Plexauridae Octocorals and the Search for Marine Natural Products from Cultured Microbes

Even though <1% of coral-associated microbes can be cultured,[94] culture-based studies still provide the best way to access microbial MNPs and to determine the roles of potential symbionts.[95,96] Similar to culture-independent studies on octocorals, extensive culture-dependent studies have not been carried out on *E. fusca* and related *Plexauridae* octocorals. Previous coral studies have isolated a high percentage of novel bacteria (20-32%),[97,98] so the probability of isolating new bacteria with unexplored chemistry is anticipated from the *Plexauridae* octocorals.

Results of previous octocoral and coral studies have also shown that a high proportion (20-70%) of cultured bacteria produce antimicrobial substances,[73,74,99-101] and novel antimicrobials with new mechanisms of action are highly desired due the rise of evolving, drug-resistant pathogens.[102-105] Thus, the search for novel antimicrobials will be of high interest in this study.

In addition to exploring cultured microbes for their ability to produce novel antimicrobials, the search for a fuscol producer from *E. fusca*-derived cultures will also be an important aspect of this study. In the marine environment, culturing the microbial producer of a marine invertebrate-derived MNP is rare, as the microbe likely requires its host's environment for survival. However, it has been achieved. The isolation of the bacterium responsible for the biosynthesis of the anti-malarial MNP, manzamine (Figure 1.1.B, p. 3), was reported in the patent literature from a *Micromonospora* sp. isolated from the sponge *Acanthostrongylophora* sp. in the absence of the sponge tissue.[106]

Chapter 3 (pp. 114-201) will explore the culture-dependent microbial community of *E. fusca* and related *Plexauridae* octocorals. In addition to characterizing the culturable microbial communities of these octocorals, this study will compare the cultured isolates to the culture-independent community to determine if any of the cultures are dominant members of the culture-independent community. Finally, microbial cultures will be further investigated for their antimicrobial activity and fuscol production.

1.2.3 Chapter 4: Formal Characterization of Endozoicomonas spp. nov.

The isolation of dominant members of the culture-independent microbial community provides the opportunity to further explore the metabolism of putative octocoral symbionts. Chapter 4 (pp 202-232) will discuss the formal characterization of the two novel *Endozoicomonas* species using a polyphasic approach. In addition to being dominant members of octocoral microbial communities,[64,65,69] *Endozoicomonas* spp. have been found to be dominant members of culture-independent coral microbial communities from around the globe.[68,93,108-112] To date, only three other *Endozoicomonas* spp. have been formally characterized (*E. elysicola*,[113] *E.*

montiporae,[114] *E. numazuensis*[115]), and these cultures were obtained from diverse marine invertebrate species (sea slug, hard coral, and sponge, respectively). *E. euniceicola* EF212^T and *E. gorgoniicola* PS125^T are the first two isolates characterized from Caribbean octocorals.

1.2.4 Chapter 5: Genome Sequencing of Endozoicomonas spp. nov. to Determine their Function in Octocorals and Other Marine-Invertebrates

Endozoicomonas spp. nov. EF212^T and PS125^T were cultured from the octocorals *E. fusca* and *Plexaura* sp., respectively, and culture-independent approaches showed that these bacteria were abundant members of the microbiome of these octocorals. Additionally, members of this bacterial genus have been reported to be prevalent in many other corals and marine invertebrates from geographically diverse locations. The abundance and ubiquity of these bacteria suggests that they may be essential to marine invertebrate health, yet their function(s) and metabolic capabilities are completely unexplored to date. Thus, we set out to determine their roles and metabolic capabilities through genome sequencing.

It has been suggested that these bacteria may provide the coral with essential nutrients,[64] cycle nutrients (*e.g.* sulfur) in the oligotrophic coral ecosystems,[71,93,116] and/or produce antimicrobial compounds.[109-110,117-120] In addition, it was of particular interest to determine if the genome of EF212^T, isolated from *E. fusca*, contained genes involved in diterpene biosynthesis. Identification of the genes responsible for the production of fuscol and related diterpenes would pave the way for future investigations into their biosynthesis. Subsequent expression of the identified biosynthetic pathway into a heterologous host could lead to a sustainable source of these diterpenes, facilitating their progression through clinical trials.

Chapter 5 (pp 233-264) will discuss the genome sequencing analysis of EF212^T and PS125^T to date.

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CHAPTER 2: A COMPREHENSIVE, CULTURE-INDEPENDENT MICROBIAL DIVERSITY ANALYSIS OF *EUNICEA FUSCA* AND RELATED *PLEXAURIDAE* OCTOCORALS

2.1 Introduction

2.1.1 The Discovery of Coral-Associated Microbes

The biological diversity of coral reefs has been compared to terrestrial rainforests.[1] This biodiversity is not only visually apparent through the rich array of macrofauna, but also through the inconspicuous assortment of microfauna. The presence of coral-associated microfauna has been known for over 40 years, with much of the early research investigating the coral dinoflagellate symbiont, *Symbiodinium* spp. In most shallow-water corals, this microbial symbiont is known to provide up to 95% of the host's carbon requirements.[2] Over the past few decades, the importance of other microbes living within corals has also become apparent. Early studies of coral-associated bacteria[3-7] and fungi,[8-17] using culture-based techniques and light microscopy, allowed scientists to study the metabolism of isolated microbes and to determine their location within the coral tissue, respectively. However, as only a minor fraction of marine microbes are culturable (<1%) on enriched media,[18-24] culture-independent analyses were needed to discern the complete microbial diversity of corals.

2.1.2 Investigation of Coral-Microbial Assemblages using Culture-Independent Technologies

The advent of culture-independent technologies,[25,26] such as clone libraries and denaturing gradient gel electrophoresis (DGGE), which molecularly analyze conserved genes or regions of DNA, allowed researchers to further explore coral-microbial assemblages. The seminal, culture-independent, coral-bacterial community study, carried out by Rohwer and colleagues (2001),[27] led many researchers to carry out similar coral-microbial diversity studies.[1,27-44] By 2010, 6,774 16S rRNA genes from 32 coral-microbial diversity studies had been deposited in GenBank.[45] In addition, culture-based, microscopy, and fluorescence *in situ* hybridization (FISH) studies[46-48] continued to be carried out to explore the metabolic functions and localization of coral-associated microbes, respectively. It was soon realized that corals did not solely function as a cnidarian and dinoflagellate symbiosis, but rather, a complex association

between many microbes and the coral host. Thus, the term 'holobiont' was coined to describe the complex interactions occurring among the coral animal and diverse microbes (dinoflagellates, bacteria, fungi, archaea, endolithic algae, protists, and viruses) that are distributed in spatially-distinct patterns (skeleton, tissue, and mucus layer of coral), but which function as a whole.[1,49,50]

This first era of culture-independent, coral-microbial studies was limited by a lack of high-throughput-sequencing (HTS). In the past few years, however, molecular techniques have progressed, and a new era of metagenomic sequencing has developed which couples polymerase chain reaction (PCR) amplification and HTS.[51,52] These pioneering next generation sequencing (NGS) technologies,[53] such as 454-pyrosequencing,[54] have been applied to study marine-microbial communities.[54-67] Pyrosequencing permits analysis of at least one order of a magnitude more individuals from microbial communities than was possible with traditional cloning and sequencing.[58,68] This allows researchers to investigate the long 'tail' of low abundance taxa that constitute the 'rare microbial biosphere'[54] of corals. Moreover, the low error rate of pyrosequencing (0.25% per sequence)[69] suggests that most of the sequences obtained after sample processing contain zero or one errors,[70] thus providing an accurate, comprehensive, and rapid picture of coral-microbial assemblages.

2.1.3 Culture-Independent Patterns Reported for Coral-Microbial Assemblages

The numerous culture-independent, coral-microbial biodiversity investigations conducted to date have led to the following conclusions:

(1) Corals harbor diverse and abundant prokaryotic communities.[1,27-29,71-75]

(2) Some corals associate with species-specific bacteria that are distinct from those in the surrounding environment (seawater, sediments, other corals, and other marine invertebrates), suggesting that corals can contribute to the structuring of their specific bacterial communities.[1,27-29,33,36,37,39,42-44,47,67,71,73,75,76-89]

(3) Although some corals maintain species-specific groups of bacteria, geographic or temporal variation may occur among the dominant bacterial group. In addition, taxonomically-related

corals (*e.g.* corals related at the family level) may share similar bacterial groups at the same geographic location, suggesting that site-specific, environmental factors can also contribute to structuring coral bacterial communities.[29,40-42,61,66,84,90-93]

(4) Differences in bacterial communities are observed between healthy and bleached/diseased corals, suggesting that environmental stressors can alter bacterial assemblages.[27-31,33,35-38,40,42,43,87,94-97]

(5) Species-specific dinoflagellate *Symbiodinium* clades have been found in some shallow-water corals,[98] and certain bacteria present in the holobiont may be regulated by the specific *Symbiodinium* clade.[90,99-101]

(6) In addition to bacteria and dinoflagellates, commensal fungi,[11,16,36,41,102] archaea,[36,74,75,103] protists,[36,104-107] and viruses[36,108-112] may also be associated with healthy corals. These groups may not be species-specific, but there may be coral-specific groups.[13,103,113-115]

2.1.4 Hypothesized Roles for Coral-Associated Microbes

As coral-microbial structure became more apparent, the functions of coral-associated microbes began to be understood, as well. Some of the roles of coral-associated microbes are as follows:

(1) The coral 'hologenome' (coral and all associated genomes) may expand the ecological niche and metabolic capabilities of the coral host, providing rapid adaptive potential for the holobiont.[116,117] As the 'coral probiotic hypothesis'[49] suggests, corals can adapt to changing environmental conditions (*e.g.* water quality, light exposure, temperature, pH, and salinity) by altering specific bacterial partners that maximize the health of the coral holobiont.[1,49,116,118-120]

(2) Associated microbes may protect the coral from the invasion of potentially pathogenic microbes[121] through the production of antimicrobial compounds,[36,88,107,122-139] bioactive compounds,[140] or by occupying physical space.[127,141-147]

(3) Associated microbes may provide biogeochemical cycling of nutrients (*e.g.* nitrogen, carbon, and sulfur) and other dissolved organic matter within coral tissues and the reef

ecosystem.[5,36,86,139,146,148-159] In particular, the sulfur compound dimethylsulfoniopropionate (DMSP), which is produced in large quantities by symbiotic *Symbiodinium*[160-162] and found in significant concentrations in zooxanthellate coral,[163-168] is cycled by diverse coral-associated microbes.[117,146,159,169,170]

(4) Coral-associated microbes may remove coral metabolic waste products.[171]

(5) Certain microbes may directly serve as a food source[86] or biosynthesize essential nutrients and vitamins for the coral.[172]

(6) Associated microbes may produce quorum sensing (QS) molecules that regulate behaviors and phenotypes between microbes[173] and/or the host coral.[174,175]

2.1.5 Rationale for Investigating the Culture-Independent Microbial Community of Eunicea fusca and Related Plexauridae Octocorals

While much has been learned about coral-microbial assemblages and their functions over the past few decades, there is still much that is unknown. Furthermore, most of the culture-independent microbial diversity studies to date have focused on scleractinian corals, while only a handful have been carried out on octocorals.[32,47,61,66,92,94,176-180] Of these octocoral studies, very few have investigated the effect that different geographic environments may have on microbial composition. Thus, this study aimed to investigate the culture-independent microbial community of octocoral *Eunicea fusca* (Duchassaing & Michelotti, 1860) and related *Eunicea* sp. and *Plexaura* spp. endemic to Florida and The Bahamas. All octocorals in this study are of the order *Alcyonacea*, family *Plexauridae* and thus will be referred to as *Plexauridae* as a group throughout this study.

E. fusca was of particular interest in this study, as it is the sole source of the diterpene marine natural products (MNPs), fuscol,[181] the fuscosides,[182] eunicol,[183] and eunicidiol.[184] These diterpenes, as well as synthetic analogues, have been shown to be selective inhibitors of leukotriene synthesis in the arachidonic acid cascade, demonstrating potent and selective anti-inflammatory activity.[182,184-188] However, they cannot progress through clinical trials due to

supply issues (Chapter 1, section *1.1.4*, pp. 4-10). Recent research suggests that some microbial symbionts may contribute to the biosynthesis of some marine invertebrate natural products.[189-195] Therefore, if a stable microbial symbiont were to be found in this study, it may provide insights into the biosynthetic source of these MNPs, and if proven to be the true source, it could help to alleviate the supply issue of the MNPs and allow them to enter clinical trials.

In addition to a therapeutic application, establishing the healthy microbial consortia of these *Plexauridae* across geographic locations will enable identification of which microbial species play key roles in maintaining holobiont health. In the future, this knowledge of their healthy microbial consortia can be used to determine the health status of these *Plexauridae* living on damaged or diseased coral reefs.[1,42,67]

2.1.6 Aims of this Culture-Independent Study

This study aimed to answer the following questions. (1) What is the microbial community (bacteria, fungi, dinoflagellates, and archaea) of *E. fusca* at geographically-separate locations in Florida and The Bahamas? This question will be answered by collecting *E. fusca* samples from four geographic sites (two off the coast of Florida, USA, and two off the coast of Bimini, The Bahamas) to determine the *E. fusca* microbial consortia across geographically-separated ecosystems. (2) How do closely-related *Plexauridae* microbial communities compare at the same geographic location (The Bahamas)? This question will be answered by collecting family-related *Plexauridae* species (*Eunicea* sp. and *Plexaura* spp.) at The Bahamas sites, investigating their microbial compositions, and comparing their microbial compositions to *E. fusca*'s. (3) How do *Plexauridae* microbial communities compare to surrounding seawater microbial communities? This question will be answered by collecting surrounding seawater from all sites and comparing the microbial composition of the surrounding seawater environment to the *Plexauridae*. The culture-independent techniques of 454-pyrosequencing, DGGE, clone libraries, and species-specific microbial primers will be employed to answer these key questions.

2.2 Materials and Methods

2.2.1 Field Collection and Sample Processing

Nine *Eunicea fusca* (EF), one *Eunicea* sp. (ES), and two *Plexaura* spp. (PS1 and PS2), were collected via SCUBA diving at two sites off the coast of Florida (FL) (FL1 and FL2) and at two sites in Bimini, The Bahamas (BS) (BS3 and BS4) in June of 2009 (Figure 2.1 and Table 2.1).

Three *Plexauridae* samples were collected at each site (replicates A, B, and C) (Table 2.1). Healthy octocoral branches (~30 g) were aseptically excised from each coral colony and placed into Whirl-Paks® (Nasco, Fisher Scientific, Toronto, ON) underwater. On the surface, the samples were maintained at 18-22 ^{o}C until samples were processed (<4 h). Coral samples were aseptically cut into 0.5-1.0 cm lengths, transferred into 50 ml centrifuge tubes, and washed three times with sterile, filtered (0.22 μM Cellulose Acetate, Corning®, VWR, Mississauga, ON) seawater to remove loosely-associated surface bacteria. Approximately half of each *Plexauridae* sample was placed into individual centrifuge tubes and frozen on dry ice. Samples were shipped on dry ice and then stored at -80 ^{o}C until coral genomic DNA (gDNA) isolation was completed. At each site, 200-1000 ml of seawater was collected in Ziploc® bags (Johnson & Son, Inc., Racine, WI) (SW-FL1, 1000 ml; SW-FL2, 200 ml; SW-BS3, 200 ml; SW-BS4, 500 ml). Bacteria in surrounding seawater were collected on 0.22 μm Cellulose Acetate filters (Corning), and filters were was placed into sterile 15 ml centrifuge tubes, shipped on dry ice, and then stored frozen at -80 ^{o}C until seawater gDNA isolation was completed.

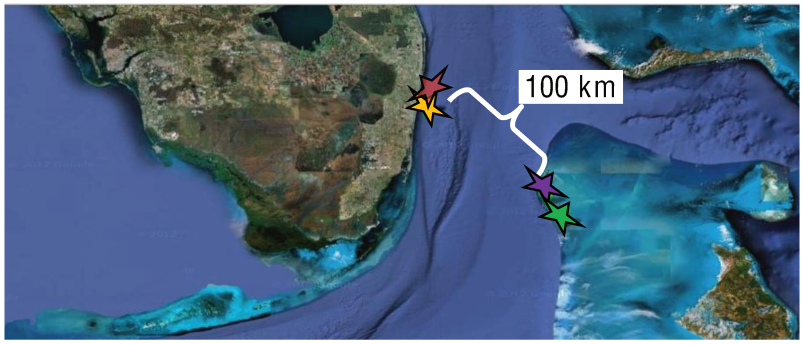


Figure 2.1 Four sample locations in Florida (FL) and The Bahamas (BS) where *Plexauridae* octocoral were collected. Image from GoogleMaps©.
Key for starred locations: Red = FL site 1; Yellow = FL site 2; Purple = BS site 3; Green = BS site 4

Table 2.1 Collection data for *Plexauridae* and seawater samples.

Site	Location (Lat., Long.; Description)	Depth (m)	Seawater Temperature (°C)	Sample Type	Sample ID	Collection Date
FL1	26°18.736'N, 80°03.583'W; South Florida Crab Cove	19.0	25	*Eunicea fusca*	EF-FL1-A	02-June-2009
					EF-FL1-B	
					EF-FL1-C	
				seawater	SW-FL1	
FL2	26°18.068'N, 80°04.112'W; Hillsboro Ledge	12.5	25	*Eunicea fusca*	EF-FL2-A	02-June-2009
					EF-FL2-B	
					EF-FL2-C	
				seawater	SW-FL2	
BS3	25°48.182'N, 79°27.033'W; Victory Reef	15.0 - 18.0	27	*Eunicea* sp.	ES-BS3-A	04-June-2009
				Eunicea fusca	EF-BS3-B	
					EF-BS3-C	
				seawater	SW-BS3	
BS4	25°31.478'N, 79°17.948'W; Tuna Alley (inside reef)	17.0	27	*Eunicea fusca*	EF-BS4-A	05-June-2009
				Plexaura spp.	PS1-BS4-B	
					PS2-BS4-C	
				seawater	SW-BS4	

Non-EF *Plexauridae* are highlighted in pink and SW samples in blue.
Abbreviations: Lat. = latitude; Long. = longitude; m = meters; ID = identity; FL = Florida; BS = The Bahamas; FL1 = Florida site 1; FL2 = FL site 2; BS3 = The Bahamas site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; SW = Seawater; A, B, C = *Plexauridae* replicates at each site

2.2.2 Plexauridae Species Identification

The identity of EF was confirmed by the detection of fuscol in coral extracts. EF is the only source of fuscol;[181] thus, the presence of this metabolite serves as a chemotaxonomic marker. *Plexauridae* samples (~1 cm) were extracted in 1 ml of dichloromethane/methanol (1:1) for 40 min, and the extracts were spotted in triplicate onto thin layer chromatography (TLC) aluminum-silica gel/UV plates (250 μm thickness, EMD Millipore, VWR, Mississauga, ON) and placed in a hexane/ethyl acetate (7:3) chamber for 5 min until the solvent front reached 1 cm from the top of the plate. To visualize separated compounds, the TLC plate was sprayed with 10% (v/v) H_2SO_4 in methanol solution, heated (~120 ^oC) for 1 min, and viewed under UV light. The presence of fuscol was confirmed by comparison to an authentic fuscol standard (high performance liquid chromatography-purified compound provided by D. Marchbank, Kerr Lab).

To further confirm the identity of the *Plexauridae* octocorals, the ITS2 region of the 18S rDNA was amplified using the octocoral-specific primers, 5.8S-436F and 28S-663R.[197] PCR amplification was carried out using the following conditions: a 1X concentration of EconoTaq 2X master mix (Lucigen, Middleton, WI), 1.0 μM of each primer, 5% (v/v) DMSO and 40 ng of template DNA. PCR cycling conditions included an initial denaturing period of 2 min at 94 ^oC, followed by 30 cycles of 94 ^oC for 30 s, 56 ^oC for 45 s, and 72 ^oC for 1 min 30 s, and a final extension of 10 min at 72 ^oC. Amplicons were analyzed by gel electrophoresis (120 V, 45 min, BioRad, Mississauga, ON) using 1.0% agarose gel (Agarose, Fisher Scientific) containing 0.001% ethidium bromide. PCR products (~250 base pairs [bp]) were visualized using a UV transilluminator (BioSpectrum®, OptiChemi HR Camera, Upland, CA). PCR products were directly sequenced using the 28S-663R (refer to section *2.2.9*, pp. 40-41) or were cloned using the GC Cloning and Amplification kit (Lucigen, Middleton, WI) following the manufacturer's protocol. Plasmid DNA was isolated from ten clones per octocoral sample using the QIAprep Spin Miniprep Kit (Qiagen, Toronto, ON), and clones containing inserts of the expected size were sequenced using the M13-49R primer (Promega, Fisher Scientific).

In addition to the chemotaxonomic (TLC) and genotypic (ITS2 sequencing) experiments, a phenotypic spicule analysis was carried out by Dr. R. Ritson-Williams at the Smithsonian Marine Station in Fort Pierce, FL to confirm the identity of the *Plexauridae* species.

2.2.3 Plexauridae gDNA Extraction

Plexauridae holobiont gDNA was extracted using a modified phenol/chloroform protocol.[197,198] Briefly, ~0.5 g of each octocoral was crushed into a fine powder using liquid nitrogen, and 5 ml of DNA Isolation Buffer (25 mM EDTA, 25 mM Tris, 0.5 M NaCl; pH 8.0) was added. Proteinase K (1 mg ml^{-1}), lysozyme (1 mg ml^{-1}), polyvinylpyrrolidone (9 g l^{-1}), and 0.5% (v/v) sodium dodecyl sulfate were added, and the mixture was incubated at 37 ^{o}C for 20 min, followed by 55 ^{o}C for 2 hr. Samples were centrifuged at 4,500 x g for 15 min (22 ^{o}C), and the supernatant was retained. The salt concentration of the supernatant was adjusted to 0.3 M with sodium acetate and then extracted with an equivalent volume of phenol/chloroform/isoamyl alcohol (25:24:1). Samples were centrifuged at 10,733 x g for 5 min (22 ^{o}C), and the aqueous layer was retained; another extraction was performed as above. Again, the aqueous layer was retained, and an equivalent volume of chloroform/isoamyl alcohol (24:1) was added, briefly vortexed, and centrifuged at 10,733 x g for 5 min (22 ^{o}C). The aqueous layer was retained, and gDNA was precipitated with 0.7 volumes of isopropanol. DNA was pelleted by centrifugation at 10,733 x g for 50 min (4 ^{o}C), and the pellet was washed with 5 ml of 70% ethanol and then air dried for 10 min. gDNA was dissolved in 200 µl of 10 mM Tris-HCl (pH 8.0). To remove potential PCR inhibitors, the *OneStep*™ PCR Inhibitor Removal Kit (Zymo Research, Irvine, CA) was used following the manufacturer's protocol, and purified gDNA was quantified on a NanoDrop Spectrophotometer (ND-1000, NanoDrop Technologies, Inc., Wilmington, DE).

2.2.4 Seawater gDNA Extraction

gDNA was isolated from bacteria filtered from the seawater samples using the UltraClean™ Water DNA Isolation Kit (Mo Bio Laboratories, Inc., Carlsbad, CA) following the manufacturer's protocol.

2.2.5 Bacterial 454-Pyrosequencing

The 16S rDNA (hypervariable regions V1, V2, and partial V3) from the *Plexauridae* and seawater samples was sequenced at Research and Testing Laboratory (RTL) (Lubbock, TX) using the bacterial Tag-Encoded FLX Amplicon Pyrosequencing (bTEFAP) method[199] based on RTL protocols (www.researchandtesting.com). Briefly, amplicons were generated from template gDNA (20 ng μl^{-1}) using the 16S rDNA 27F and 519R primers,[200] and amplicons were pyrosequenced from the 27F end. 16S rDNA Sequences were initially processed at RTL to remove failed sequences, low quality sequence 3' or 5' ends (with numerous N's), pyrosequencing sequence tags, non-bacterial sequences, chimeras, and sequences <150 bp. Sequence files were further processed in-house using the Ribosomal Database Project (RDP) release 10, update 3 (http://pyro.cme.msu.edu/).[201] Using the RDP, 27F and 519R primers were trimmed from the sequences, and sequences <250 bp were removed from the sample libraries. Sequences were then aligned, clustered, and taxonomically classified using the RDP Classifier (50 % confidence threshold[346]). Sequences that could not be assigned to a taxon using the 50% confidence threshold were displayed under an artificial 'unclassified' taxon. A genus-level composition graph of the RDP Classifier data was created using Microsoft® Excel, including groups that made up ≥0.5% of the composition of each sample (groups or singletons that constituted <0.5% of the total community were grouped into a category labeled 'Other').

Using the RDP, sequences were statistically analyzed using richness (observed operational taxonomic units [OTUs] and Chao1 estimate) and diversity (Shannon diversity and equitability) indices (97% sequence similarity cut-off). Sequences were dereplicated (97% sequence similarity cut-off) within each *Plexauridae* and seawater sample. To confirm the RDP Classifier taxonomic grouping and to taxonomically identify (to the species level) the most abundant sequences, the three most abundant OTUs in each sample were searched using the National Center for Biotechnology Information ([NCBI], Bethesda, MD) Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov)[202] with the BLASTn algorithm in the

Nucleotide Collection (nr/nt) (excluding uncultured/environmental sample sequences) GenBank database.

Using PRIMER5,[347] the Bray-Curtis similarity equation (with square root transformation) was used to construct a similarity matrix between the *Plexauridae* and seawater samples based on their generic-level taxonomic composition. This similarity matrix was then used to construct a single linkage cluster analysis and nonmetric multidimensional scaling (nMDS) plot to determine how similar the samples were to each other based on their generic composition.

2.2.6 Bacterial and Fungal Denaturing Gradient Gel Electrophoresis (DGGE)

16S rDNA DGGE was used to confirm the most abundant bacterial groups detected by 454-pyrosequencing. PCR amplification of the 16S rRNA gene was done using the universal Eubacteria 16S rDNA primers 27F and 1525R[200] using the same reaction conditions previously described for amplification of the ITS2 (section *2.2.2*, p. 35). PCR cycling conditions included an initial denaturing period of 3 min at 95 °C, followed by 35 cycles of 95 °C for 45 s, 54 °C for 1 min, and 72 °C for 1 min 30 s, and a final extension of 10 min at 72 °C. A nested PCR reaction was then performed with the primers 27F-GC and 534R,[203] using a 1:100 dilution of the initial PCR amplification products as the template DNA. The same reaction conditions were used as described above, and the nested PCR cycling conditions followed a touchdown protocol with an initial denaturing period of 5 min at 95 °C, followed by 10 cycles of 95 °C for 1 min, 66 °C (decreasing by 1 °C each cycle) for 1 min, and 72 °C for 2 min. Another 20 cycles of 95 °C for 1 min, 56 °C for 1 min 30 s, and 72 °C for 2 min followed with a final extension of 7 min at 72 °C.

ITS DGGE was carried out to investigate the fungal diversity associated with the *Plexauridae* and seawater. The initial ITS fungal PCR amplification reactions used the fungal-specific primers ITS-1f and ITS-4.[204-206] The PCR reaction conditions were the same as described for the ITS2 octocoral PCR amplification (section *2.2.2*, p. 35). PCR cycling conditions included an initial denaturing period of 2 min at 95 °C, followed by 35 cycles of 95 °C for 1 min, 54.6 °C

for 1 min 30 s, and 72 °C for 2 min, and a final extension of 5 min at 72 °C. A nested PCR was performed as described above except using the primers ITS-1f-GC and ITS-2.[206,207]

16S rDNA and ITS PCR products were separated using a D-Code Universal Mutation Detection DGGE system (BioRad). Samples were loaded onto a 6% (16S rDNA amplicons) or 8% (ITS amplicons) acrylamide gel and run with 1xTAE buffer (0.04 M Tris base, 0.02 M sodium acetate, and 1 mM sodium EDTA; pH 7.4) using a 40-70% linear denaturing gradient of urea and formamide. Electrophoresis was run at 60 V for 24 hr (60 °C). After electrophoresis, the gels were stained for 30 min with ethidium bromide in 1xTAE buffer, rinsed with 1xTAE buffer, and visualized using a UV transilluminator. Distinct bands were excised from the DGGE gels and placed in 30 µl sterile water overnight to elute DNA. Excised bands were amplified with nested PCR primers using the same conditions as described above and re-run on the DGGE system to ensure purity and stable mobility of the bands. Bands were excised once again, and DNA was eluted and either directly sequenced (section *2.2.9*, pp. 40-41) (for bacteria only) using the primers 16S534R or cloned and sequenced (for both bacteria and fungi) as previously described (section *2.2.2*, p. 35).

Cluster analyses of the 16S rDNA DGGE and ITS DGGE gel profiles were conducted using BioNumerics version 5.0 gel compare software (Applied Maths, Austin, TX). For the 16S rDNA DGGE profiles, the parameters were as follows: Dice Similarity Coefficient, Optimization (0%), Band Matching – Min. height (0%), Min. surface (0%), Band filtering – Tolerance (1%), Tolerance change (1%), Include Uncertain bands, Complete Linkage Clustering. The same parameters were used for the ITS DGGE profiles, except the options Ignore Uncertain bands and Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering were used.

2.2.7 Symbiodinium Clade Characterization

The clade of *Symbiodinium* present in each *Plexauridae* was determined through PCR amplification and clone library construction. The 28S rRNA gene was PCR amplified using *Symbiodinium*-specific primers (28S-F and 28S-R) using conditions previously described.[208-210]

The *Symbiodinium* amplified PCR product (~650 bp) was excised from a 2% agarose gel, gel purified using Fermentas Silica Bead DNA Gel Extraction Kit (Thermo Scientific, Ottawa, ON), and cloned using either the CloneJET PCR Cloning Kit (Thermo Scientific) or the InsTAclone PCR Cloning kit (Thermo Scientific) following the manufacturers' protocols. Plasmid DNA was isolated from ten clones per sample using the EZNA® Plasmid Mini Kit I (Omega Bio-Tek, VWR), and clones containing inserts of the expected size were sequenced (refer to section *2.2.9*, pp. 40-41) using the pJET1.2R primer (Thermo Scientific).

2.2.8 Archaeal Presence

To test for the presence of archaea in the *Plexauridae*, archaeal-specific primers (Ar20F and Ar958R) were used following a previously described protocol.[211]

2.2.9 Sequencing and Phylogenetic Analysis

All PCR amplicons and clones were sequenced by Génome Québec (Montréal, QC) or Eurofins MWG Operon (Huntsville, AL). Taxonomic affiliations of sequences were determined by comparison to sequences in GenBank using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov).[202] Sequences were grouped into OTUs (97% sequence similarity cut-off) using ContigExpress (Vector NTI Advance 10.3.0, Invitrogen, Carlsbad, CA), and multiple sequences of closely-related microbes were aligned using BioEdit version 7.0.5.3.[212] Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.[213] The evolutionary history of *Endozoicomonas* and *Mycoplasma* relatives were inferred using multiple methods: Minimum Evolution (ME),[214] Maximum-Parsimony,[215] UPGMA,[216] and Neighbor-Joining (NJ),[217] and a similar topology was obtained in all phylogenetic trees. For the *Endozoicomonas* phylogenetic tree, the evolutionary history of 78 *Endozoicomonas* relatives was inferred using the ME method.[214] Bootstrap analysis was performed with 1000 resamplings.[218] The evolutionary distances were computed using the Jukes-Cantor method[219] and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm[220] at a search level of 1. The NJ algorithm[217] was used to generate

the initial tree. All positions containing gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option). There were a total of 456 positions in the final dataset. For the *Mycoplasma* phylogenetic tree, the evolutionary history of 88 *Mycoplasma* relatives was inferred using the NJ method.[217] The optimal tree with the sum of branch length = 2.70814919 is shown. Bootstrap analysis, evolutionary distances, and search were performed as described for the *Endozoicomonas* ME tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 261 positions in the final dataset.

2.2.10 Nucleotide Sequence Accession Numbers

All octocoral ITS2 (KC491237-KC491247), *Symbiodinium* 28S (KC491248-KC491268), bacteria 16S rDNA DGGE (KC491269-KC491329), fungi ITS1 DGGE (KC491330-KC491368) sequences, and the most abundant bacteria 16S rDNA 454-pyrosequences in each sample (KC511126-KC511203) were deposited in GenBank under the indicated accession numbers. All 454-pyrosequencing sequences were deposited in the NCBI Short Read Archive under the accession number SRA059231.

2.3 Results

2.3.1 Plexauridae Identification

TLC (Figure 2.2) and ITS2 sequencing confirmed that nine out of 12 of the *Plexauridae* samples were *E. fusca* (99% sequence identity to *E. fusca* ITS2, GenBank Accession No. EF490983).

The ITS sequences from the three non-EF samples indicated that ES-BS3-A was most closely-related (88% sequence identity) to *Eunicea* sp. JAS-2007-1 ITS2 (GenBank Accession No. EF490976), and PS1-BS4-B and PS2-BS4-C were most closely-related (93% and 99% sequence identity, respectively) to *Plexaura homomalla* ITS (GenBank Accession No. EF490974). The ITS identification of all *Plexauridae* octocorals was consistent with the spicule analysis. *Plexauridae* species identity and sample identity (ID) are listed in Table 2.1 (p. 34)

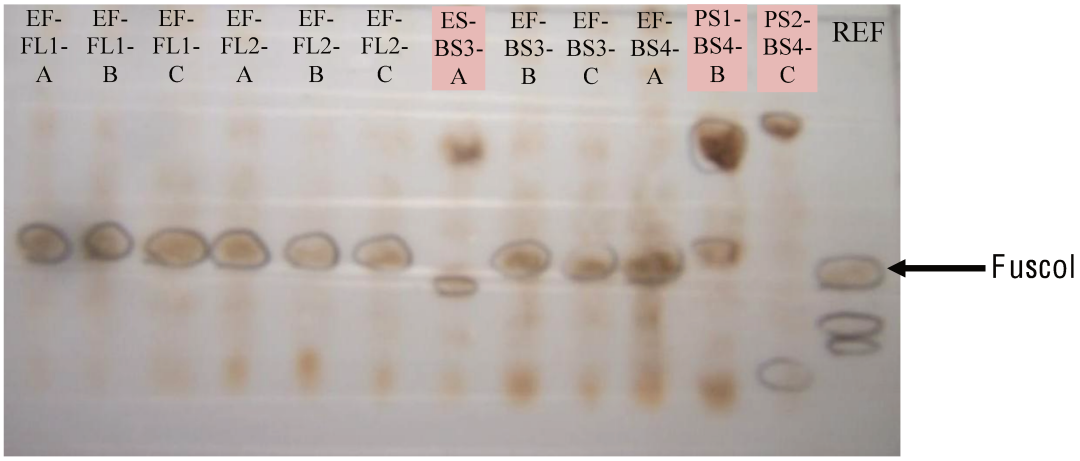


Figure 2.2 Thin layer chromatography of *Plexauridae* spp. to identify *Eunicea fusca* (EF). Non-EF *Plexauridae* spp. are highlighted in pink.
Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; REF = reference fuscol

2.3.2 Composition of Bacteria in Plexauridae and Surrounding Seawater using 454-Pyrosequencing and DGGE

2.3.2.1 Diversity Statistics of *Plexauridae* and Surrounding Seawater Bacterial Communities using 454-Pyrosequencing

After quality filtering of the 454-pyrosequencing data, a total of 148,671 sequences were recovered from the 12 *Plexauridae* octocorals (113,050 sequences) and four surrounding seawater samples (35,621 sequences). The average number of sequences per *Plexauridae* was 9,421, and for seawater, 8,905, with an average sequence length of 472 bp for the *Plexauridae* and 455 bp for the seawater.

The number of OTUs and the estimated species richness and diversity indices (97% sequence similarity cut-off) are listed in Table 2.2. The highest number of OTUs in *Plexauridae* was found in PS1-BS4-B and the lowest in EF-BS3-C, with an average of 139 OTUs in *Plexauridae* (FL *Plexauridae* average = 149 OTUs; BS *Plexauridae* average = 130 OTUs; EF average = 141 OTUs; non-EF average = 134 OTUs). For the seawater, the FL sites had more OTUs (average = 1,040) than the BS sites (average = 519). Among the *Plexauridae*, sample ES-BS3-A was the most diverse (H' = 2.56), and sample EF-FL1-A was the least diverse (H' = 1.52). For the seawater, the BS sites were more diverse (average H' = 4.92) than the FL sites (average H' = 4.38). When calculating evenness, the *Plexauridae* (average E' = 0.43) were less even than the seawater (average E' = 0.71).

Table 2.2 Summary of *Plexauridae* and seawater 454-pyrosequencing diversity statistics.

Sample ID (SRA Accession No.)	No. Sequences	Avg. Sequence Length	No. OTUs[a]	Chao1 Richness[a]	Shannon Diversity Index (H')[a]	Shannon Equitability Index (E')[a]	% Singletons[b]
EF-FL1-A (SRR650232)	8989	468	102	125	1.52	0.33	29.4
EF-FL1-B (SRR650233)	11616	485	132	204	2.17	0.44	37.9
EF-FL1-C (SRR650234)	11316	471	183	262	2.05	0.39	37.2
EF-FL2-A (SRR650235)	13314	463	148	201	2.22	0.44	29.7
EF-FL2-B (SRR650236)	8856	466	197	301	2.11	0.40	39.6
EF-FL2-C (SRR650238)	9030	470	134	167	2.09	0.43	28.4
EF-BS3-B (SRR650316)	5058	473	151	182	2.52	0.50	29.8
EF-BS3-C (SRR650330)	744	476	37	60	1.56	0.43	45.9
EF-BS4-A (SRR650345)	12519	464	186	242	2.38	0.46	26.3
ES-BS3-A (SRR650239)	4380	463	90	131	2.56	0.57	28.9
PS1-BS4-B (SRR650346)	11676	487	206	264	2.24	0.42	32.5
PS2-BS4-C (SRR650348)	15552	475	107	164	1.82	0.39	36.4
SW-FL1 (SRR650350)	12433	456	1070	1689	4.39	0.63	46.1
SW-FL2 (SRR650351)	10460	456	1011	1527	4.36	0.63	45.0
SW-BS3 (SRR650369)	4387	454	339	489	4.79	0.82	32.4
SW-BS4 (SRR650370)	8341	453	700	941	5.04	0.77	33.9
Avg. *Plexauridae*	9421	472	139	192	2.10	0.43	33.5
Avg. Seawater	8905	455	780	1161	4.65	0.71	39.3

[a]All determined at 97% sequence similarity cut-off.
[b]In dereplicated sequence libraries (97% similarity cut-off).
Non-EF *Plexauridae* are highlighted in pink and SW samples in blue.
Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater; ID = identity; SRA = sequence read archive; SRR = sequence read run; Avg. = average; No. = number; OTUs = operation taxonomic units

2.3.2.2 Phylogenetic Affiliations of *Plexauridae* and Surrounding Seawater Bacterial Communities using 454-Pyrosequencing

At a 50% confidence threshold, 97.4% of the quality filtered 16S rDNA sequences could be assigned to a known bacterial phylum using the RDP Classifier. Twenty-two phyla were recovered from the *Plexauridae* bacterial communities with an average of ten phyla per octocoral. The only ubiquitous phylum among all *Plexauridae* was *Proteobacteria*. Among EF, there was an average of 11 phyla, with the four ubiquitous phyla *Proteobacteria*, *Tenericutes*, *Firmicutes*, and *Cyanobacteria*. At the class level, three classes were shared between all *Plexauridae* (*Gammaproteobacteria*, *Alphaproteobacteria*, and *Clostridia*), and EF contained six ubiquitous classes (*Mollicutes*, *Cyanobacteria*, and *Bacilli*, in addition to the three aforementioned classes). At the genus level (Figure 2.3), *Endozoicomonas* was the only genus found in all *Plexauridae*. All EF contained this genus as well as *Mycoplasma* relatives and 'unclassified' bacteria in the family *Oceanospirillales*.

In the seawater, 23 phyla were detected with an average of 15 phyla per sample. The Floridian SW had more phyla (average = 18) than the Bahamian SW (average = 13). Seven phyla were ubiquitous among all SW samples (*Verrucomicrobia*, *Actinobacteria*, *Bacteroidetes*, *Firmicutes*, *Cyanobacteria*, *Tenericutes*, and *Proteobacteria*). Ten classes were shared between all SW samples (*Cyanobacteria*, *Alpha-*, *Beta-*, *Gamma-*, and *Delta-proteobacteria*, *Actinobacteria*, *Clostridia*, *Sphingobacteria*, *Flavobacteria*, and *Opitutae*). 36 genera were ubiquitous among SW samples, but the communities were mainly dominated by *GpIIa* and *Pelagibacter* (Figure 2.3).

The three most abundant OTUs (Table 2.3) for each *Plexauridae* and seawater sample revealed that *Endozoicomonas*, related *Hahellaceae* bacteria ("*Loripes lacteus* sulfur-oxidizing, gill-symbiont clone 1B"), and *Mycoplasma* or *Spiroplasma* relatives were the prominent members of *Plexauridae* libraries, whereas the seawater libraries were dominated by *GpIIa* (*Prochlorococcus* and *Synecococcus*) and *Pelagibacter*.

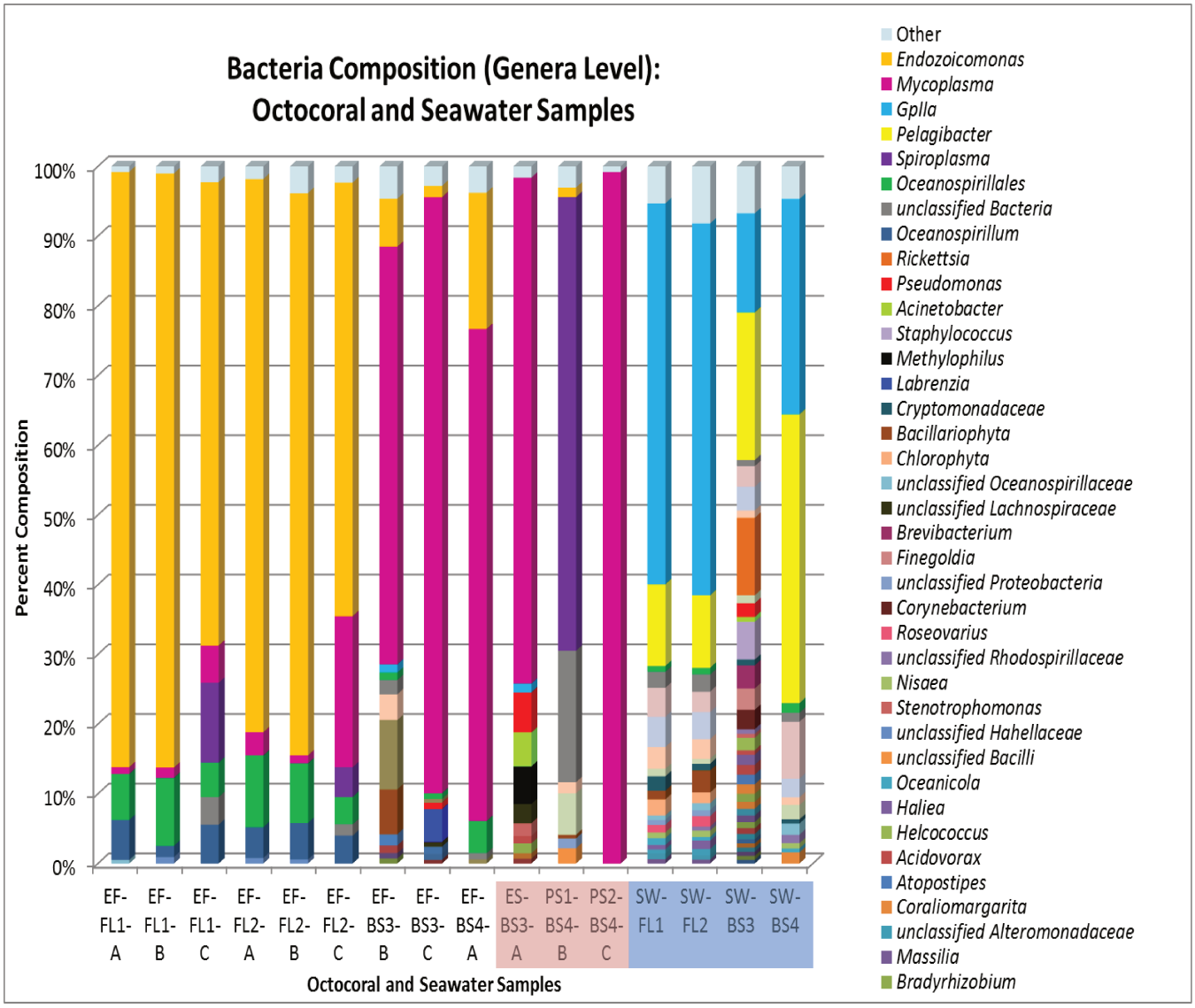


Figure 2.3 Genus-level bacteria composition (using 454-pyrosequencing) of *Plexauridae* and seawater samples. All genus-level operational taxonomic units (OTUs) ≥0.5% are represented on the graph; those OTUs <0.5% are grouped in the category 'Other.' Bacteria that could not be classified to the genus-level are classified at the highest identifiable taxonomic level (*i.e.* phyla, class, order, or family). Non-EF *Plexauridae* are highlighted in pink and SW samples in blue. Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater

Table 2.3 Top three dereplicated (97% sequence similarity cut-off) bacteria sequences from 454-pyrosequencing data for each sample and closest GenBank matches.

Sample ID	GenBank Accession No. of Representative Sequence (Rep. Seq.)	% of OTU in Sample	Sequence Length (bp) of Rep. Seq.	Closest GenBank BLAST Result (Accession No.)	Max ID (%)
EF-FL1-A	KC511126	68.7	401	*Endozoicomonas* sp. EF212 (JX488684)	99
	KC511127	6.7	402	*Endozoicomonas* sp. EF212 (JX488684)	99
	KC511128	6.1	411	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	94
EF-FL1-B	KC511131	40.7	250	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	92
	KC511132	16.0	336	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	94
	KC511133	15.2	401	*Endozoicomonas* sp. EF212 (JX488684)	99
EF-FL1-C	KC511136	56.4	409	*Endozoicomonas* sp. EF212 (JX488684)	98
	KC511137	11.3	401	*Spiroplasma velocicrescens* MQ-4 (NR025713)	82
	KC511138	4.4	260	*Endozoicomonas* sp. EF212 (JX488684)	99
EF-FL2-A	KC511141	38.8	405	*Endozoicomonas* sp. EF212 (JX488684)	98
	KC511142	22.0	258	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	92
	KC511143	13.4	410	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	94
EF-FL2-B	KC511146	52.6	419	*Endozoicomonas* sp. EF212 (JX488684)	98
	KC511147	13.2	245	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	92
	KC511148	10.5	415	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	94
EF-FL2-C	KC511151	47.8	397	*Endozoicomonas* sp. EF212 (JX488684)	98
	KC511152	12.4	396	*Mycoplasma zalophi* (AF493543)	87
	KC511153	7.2	388	*Mycoplasma gallinarum* ATCC15319 (JN935884)	88
EF-BS3-B	KC511160	37.1	430	*Mycoplasma vulturii* Gb-V33 (AY191226)	86
	KC511161	16.6	335	*Spiroplasma phoeniceum* P40 (NR043178)	85
	KC511162	13.2	419	*Mycoplasma vulturii* Gb-V33 (AY191226)	86
EF-BS3-C	KC511165	48.7	370	*Mycoplasma zalophi* (AF493543)	87
	KC511166	33.7	420	*Mycoplasma vulturii* Gb-V33 (AY191226)	86
	KC511167	2.7	403	*Labrenzia* sp. V7 (JX407223)	99
EF-BS4-A	KC511170	26.2	424	*Mycoplasma vulturii* Gb-V33 (AY191226)	87
	KC511171	13.8	409	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	95
	KC511172	5.6	390	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	94
ES-BS3-A	KC511155	40.3	423	*Mycoplasma vulturii* Gb-V33 (AY191226)	87
	KC511156	11.2	464	*Mycoplasma vulturii* Gb-V33 (AY191226)	85
	KC511157	8.0	394	*Mycoplasma spermatophilum* NC011720 (AF013996)	87

PS1-BS4-B	KC511174	51.4	424	*Spiroplasma velocicrescens* MQ-4 (NR025713)	81
	KC511175	9.4	363	*Spiroplasma velocicrescens* MQ-4 (NR025713)	82
	KC511176	7.7	394	*Spiroplasma velocicrescens* MQ-4 (NR025713)	81
PS2-BS4-C	KC511179	49.0	354	*Mycoplasma vulturii* Gb-V33 (AY191226)	86
	KC511180	20.3	419	*Mycoplasma vulturii* Gb-V33 (AY191226)	87
	KC511181	11.0	489	*Mycoplasma vulturii* Gb-V33 (AY191226)	85
SW-FL1	KC511184	25.4	377	*Synechococcus* sp. CC9605 (CP000110)	100
	KC511185	9.5	456	*Prochlorococcus marinus* MIT 9301 (CP000576)	99
	KC511186	3.7	380	*Pelagibacter ubique* HTCC1002 (AF510192)	99
SW-FL2	KC511189	29.9	383	*Synechococcus* sp. UW122 (JQ421035)	100
	KC511190	3.9	378	*Prochlorococcus marinus* MIT 9301 (CP000576)	99
	KC511191	3.9	449	*Synechococcus* sp. UW122 (JQ421035)	99
SW-BS3	KC511194	7.2	449	*Rickettsia massiliae* AZT80 (CP003319)	99
	KC511195	6.9	353	*Prochlorococcus marinus* MIT 9301 (CP000576)	99
	KC511196	2.2	364	*Synechococcus* sp. WH8102 (BX569694)	100
SW-BS4	KC511199	12.0	384	*Prochlorococcus marinus* MIT 9301 (CP000576)	99
	KC511200	4.3	431	*Prochlorococcus marinus* MIT 9301 (CP000576)	99
	KC511201	4.1	369	Alpha proteobacterium SCGC AAA298-C20 (HQ675220)	99

Non-EF *Plexauridae* are highlighted in pink and SW samples in blue.
Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater; ID = identity; bp = base pair; No. = number; OTU = operational taxonomic unit

2.3.2.3 Comparison of 454-Pyrosequencing Bacterial Communities Based on Geographic Location (FL vs. BS)

Even though particular taxa were common to all EF samples, the abundance of these groups varied considerably between collection sites. Floridian EF were dominated by *Endozoicomonas* (EF-FL average composition = 76.5%; EF-BS average composition = 4.9%), while the Bahamian EF were dominated by *Mycoplasma* relatives (EF-FL average composition = 5.7%; EF-BS average composition = 64.6%) (Figure 2.3, p. 47).

Similar to the octocorals, the composition of seawater bacterial communities varied between geographic locations. In Floridian SW, the cyanobacteria *GpIIa* dominated (SW-FL average composition = 54.0%; SW-BS average composition = 22.5%), while in SW-BS, the Alphaproteobacteria *Pelagibacter* dominated (SW-FL average composition = 11.1%; SW-BS average composition = 31.3%) (Figure 2.3, p. 47).

2.3.2.4 Comparison of 454-Pyrosequencing Bacterial Communities Based on *Plexauridae* Host Species at Similar Geographic Locations (The Bahamas Only)

All Bahamian *Plexauridae* samples were dominated by *Mycoplasma* or *Spiroplasma* relatives (Figure 2.3, p. 47). However, there were differences observed in the bacterial communities of the different *Plexauridae* species. Bahamian EF contained *Endozoicomonas* and related 'unclassified' *Hahellaceae* bacteria, whereas the non-EF contained *Endozoicomonas*, but in very low abundances compared to the EF, and lacked related *Hahellaceae* bacteria. In addition, ES-BS3-A (*Eunicea* sp.) had a more diverse and even bacterial community than all EF-BS (Table 2.2, p. 45; Figure 2.3, p. 47). PS1-BS4-B (*Plexaura* sp. 1) had a completely different bacterial composition than all EF, dominated by *Spiroplasma* relatives, and PS2-BS4-C (*Plexaura* sp. 2) was almost entirely dominated by *Mycoplasma* relatives, with few other dominant OTUs (Figure 2.3, p. 47).

2.3.2.5 Comparison of 454-Pyrosequencing Bacterial Communities between *Plexauridae* and Seawater Samples

The seawater bacterial communities had differing compositions than the *Plexauridae* and were dominated by *Gp1la* and *Pelagibacter*. They also had a greater species richness, diversity, and evenness than the *Plexauridae* (Table 2.2, p. 45; Figure 2.3, p. 47).

2.3.2.6 Cluster Analysis of 454-Pyrosequencing Samples Based on Bacterial Phylogenetic Affiliations

Cluster analysis of the 454-pyrosequencing taxonomic data showed that *Plexauridae* and seawater samples grouped by sample type (*Plexauridae* host species or seawater) and geographic location (FL or BS) (Figure 2.4). Similarly, an nMDS plot of the octocoral and seawater samples showed a clustering of the EF-FL samples, a broad clustering of the BS-*Plexauridae* samples, and a grouping of the seawater samples (Figure 2.5).

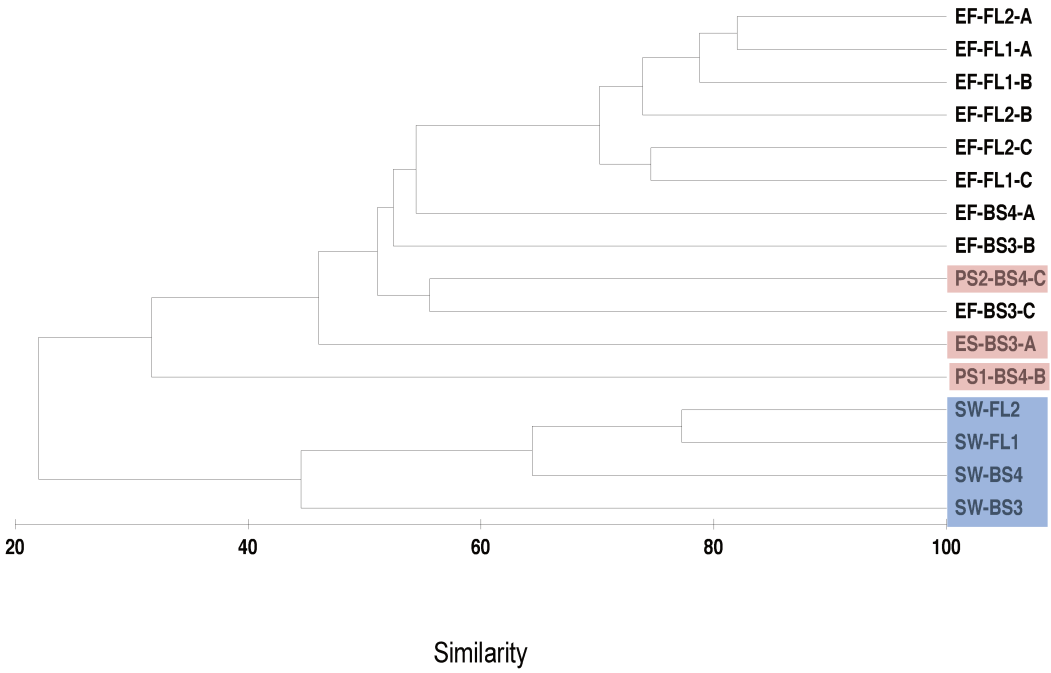


Figure 2.4 Single linkage cluster analysis of bacteria composition (using 454-pyrosequencing) from *Plexauridae* and seawater (SW) samples. Non-EF *Plexauridae* are highlighted in pink and SW samples in blue.
Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater

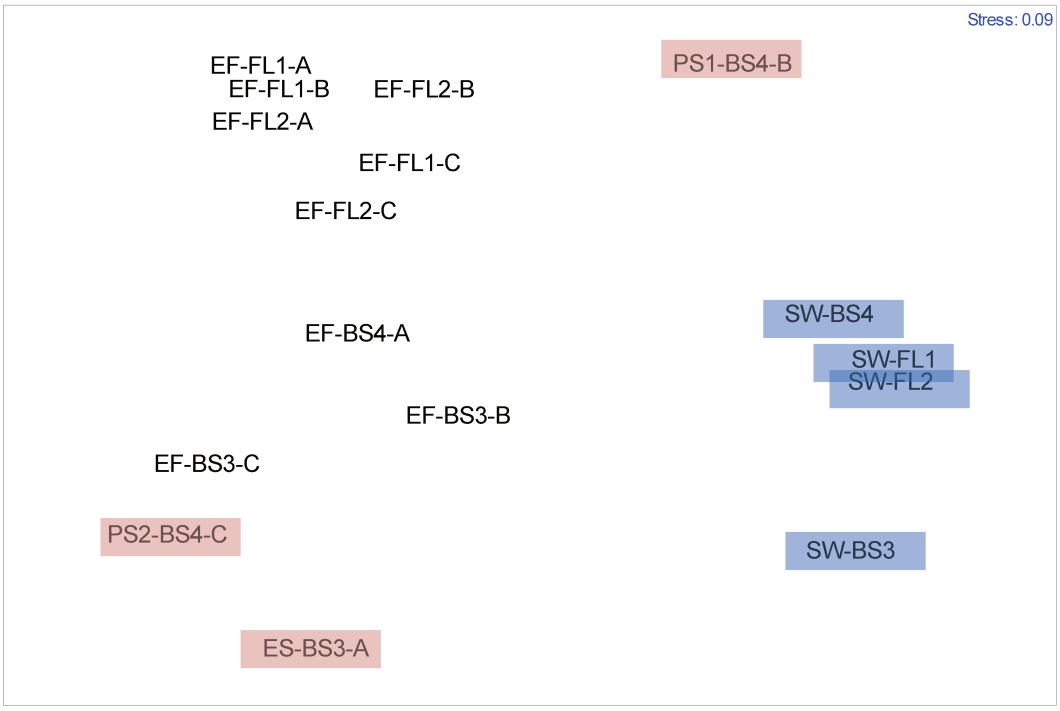
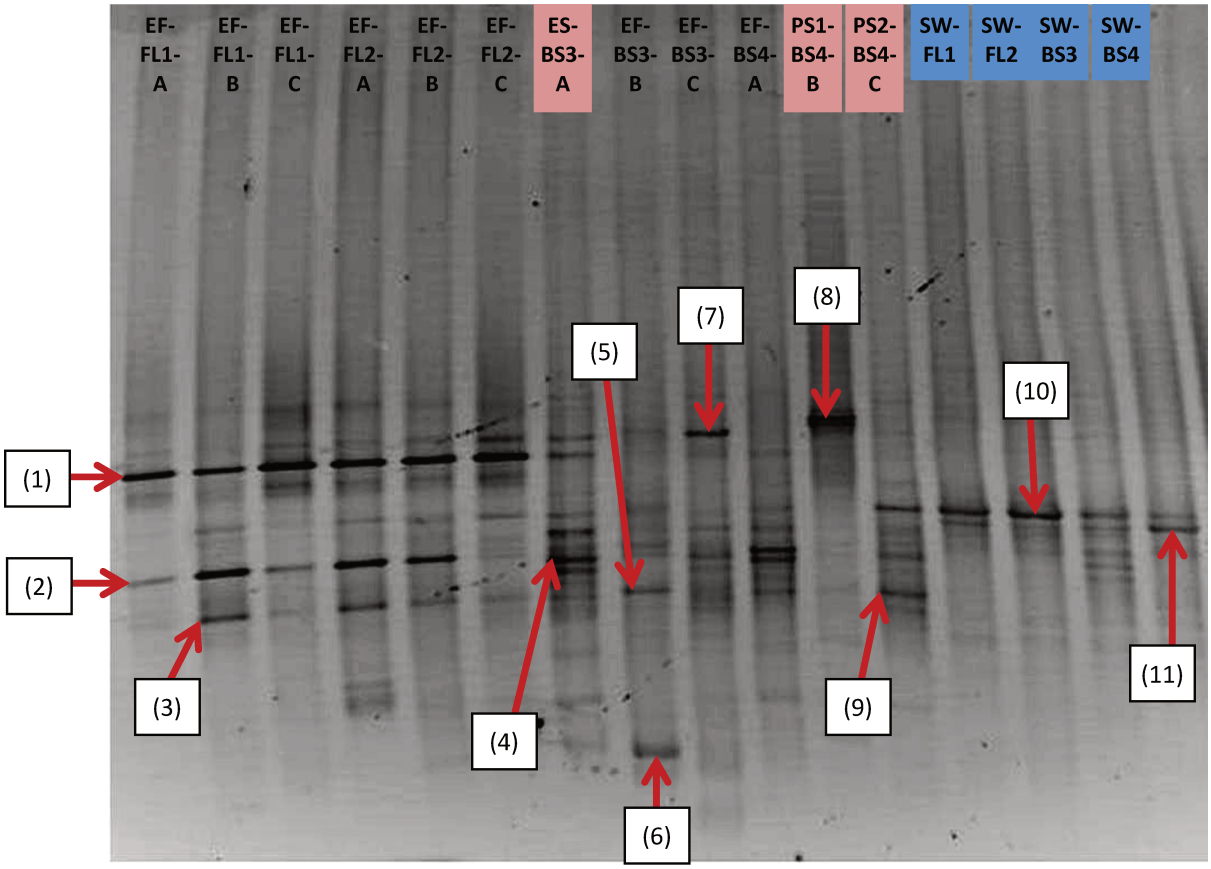


Figure 2.5 Nonmetric multidimensional scaling (nMDS) plot of bacteria composition (using 454-pyrosequencing) of *Plexauridae* and seawater (SW) samples. Non-EF *Plexauridae* are highlighted in pink and SW samples in blue.
Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater

2.3.2.7 Composition of *Plexauridae* and Surrounding Seawater Bacterial Communities using DGGE

16S rDNA DGGE fingerprinting (Figure 2.6) confirmed the geographic heterogeneity observed in the 454-pyrosequencing analysis (Figure 2.3, p. 47). The dominant *Endozoicomonas* relatives in the EF-FL DGGE fingerprints (Figure 2.6, bands 1, 2, and 3) were also the same most dominant sequences found in the EF-FL 454-pyrosequencing libraries (Table 2.3, pp. 48-49). ES-BS3-A appeared to have more bands, and hence greater diversity, supporting the 454-pyrosequencing diversity statistics (Table 2.2, p. 45). *Mycobacterium* sequences were only observed in PS1-BS4-B in both the 454-pyrosequencing and DGGE libraries (Figure 2.6, band 8). *Streptomyces* sequences were only observed in sample EF-BS3-B in both libraries (Figure 2.6, band 6), as well. *Brevundimonas* bacteria, detected in DGGE sample EF-BS3-B (Figure 2.6, band 5), were most abundant in this octocoral's 454-pyrosequencing library compared to other libraries. In the seawater, the *Gpl/a* groups, *Synechococcus* (Figure 2.6, band 10) and *Prochlorococcus* (Figure 2.6, band 11), were found to be dominant in both the DGGE and pyrosequencing libraries. Clustering based on DGGE profiles revealed the EF-FL samples grouped, the BS-*Plexauridae* samples loosely grouped, and the seawater samples grouped (Figure 2.7).



Band No.	Acc. No.	Closest GenBank Match (Acc. No.)	Max ID (%)
1	KC491269	*Endozoicomonas* sp. EF212 (JX488684)	99
2	KC491306	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	94
3	KC491271	*Loripes lacteus* gill symbiont clone 1B, sulfur-oxidizer (GQ853556)	92
4	KC491313	*Sphingobium* sp. (HM243762)	96
5	KC491315	*Brevundimonas* sp. (HQ830178)	99
6	KC491272	*Streptomyces* sp. (KC121254)	100
7	KC491291	*Chroococcales* sp. (EU259177)	93
8	KC491324	*Mycobacterium* sp. (HE663067)	97
9	KC491329	*Endozoicomonas* sp. (AB695089)	96
10	KC491273	*Synechococcus* sp. (CP000110)	100
11	KC491274	*Prochlorococcus marinus* (CP000576)	99

Figure 2.6 16S rDNA denaturing gradient gel electrophoresis (DGGE) gel of *Plexauridae* and seawater samples. Non-EF *Plexauridae* are highlighted in pink and SW samples in blue. Prominent identified bands and closest GenBank relatives are listed in the accompanying table. Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater; Acc. No. = Accession number; ID = identity

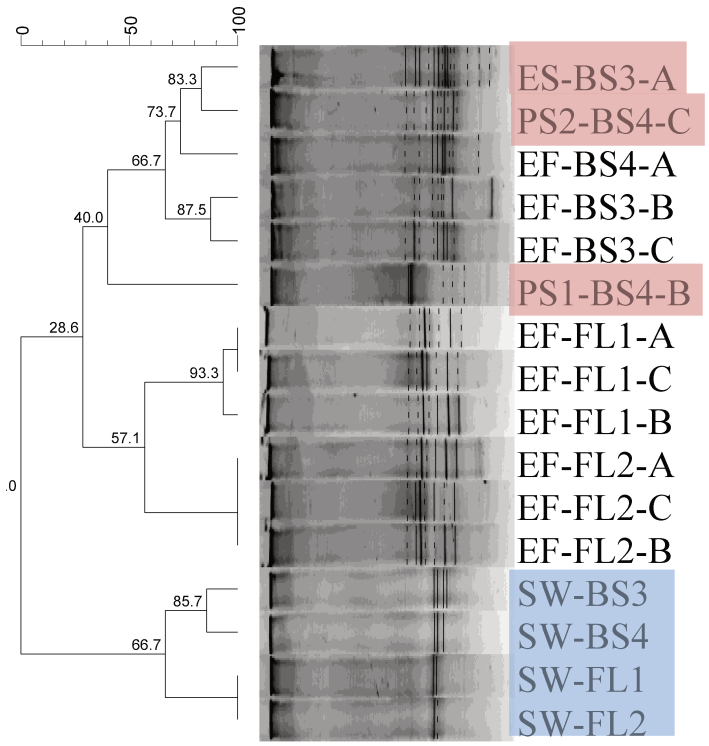
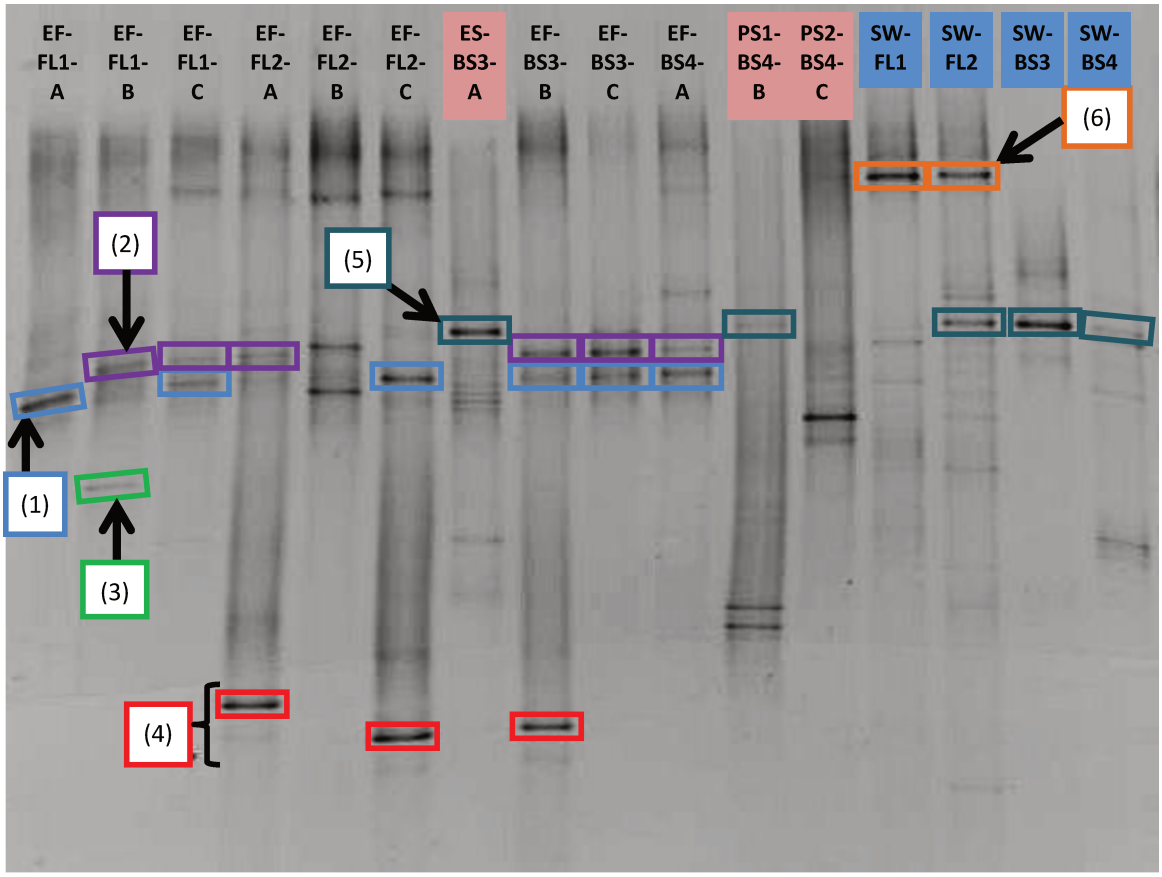


Figure 2.7 Complete linkage cluster analysis of 16S rDNA denaturing gradient gel electrophoresis (DGGE) gel. Non-EF samples are highlighted in pink and SW samples in blue. Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater

2.3.3 Composition of Fungi in Plexauridae and Surrounding Seawater using DGGE

Fungal ITS DGGE (Figure 2.8) revealed less diversity and fewer similarities between sites than the 16S rDNA DGGE of *Plexauridae* and seawater samples. However, there were a few sequences shared between EF from different sites, including a fungus 97% related to a *Toxicocladospirum* sp. (Figure 2.8, band 1) in EF-FL1-A, -C, EF-FL2-C, EF-BS3-B, -C, and EF-BS4-A, an *Ascomycota* fungus 80% related to a *Cylindrocarpon* sp. (Figure 2.8, band 4) in EF-FL2-A, -C, and EF-BS3-B, and a fungus 96% related to a *Scedosporium* sp. (Figure 2.8, band 2) in EF-FL1-B, -C, EF-FL2-A, EF-BS3-B, -C, and EF-BS4-A. EF-FL1-B contained a unique sequence 99% related to a *Hypocreales* sp. (Figure 2.8, band 3). The non-EF fungal DGGE profiles differed from the EF, lacking members of these genera. ES-BS3-A and PS1-BS4-B also shared a sequence 99% related to a *Cadophora* sp. (Figure 2.8, band 5). The seawater fungal DGGE profiles differed from the *Plexauridae*; however, the same *Cadophora* sp. found in the non-EF octocorals was found in both SW-BS samples and SW-FL2 (Figure 2.8, band 5). An uncultured fungus (<79% ITS sequence similarity) (Figure 2.8, band 6) was found in both SW-FL samples. Clustering of the fungal ITS DGGE fingerprint profiles showed that many of the EF samples grouped together (Figure 2.9).



Band No.	Accession No.	Closest GenBank Match	Sequence ID (%)
1	KC491357	*Toxicocladosporium* sp. (FJ791135)	97
2	KC491351	*Scedosporium* sp. (FJ345358)	96
3	KC491330	*Hypocreales* sp. (DQ682584)	99
4	KC491359	*Ascomycota* sp. (GU934599)	80
5	KC491338	*Cadophora* sp. (JQ796752)	99
6	KC491368	Uncultured fungus (GU942303)	79

Figure 2.8 ITS denaturing gradient gel electrophoresis (DGGE) fungal composition of *Plexauridae* and seawater samples. Non-EF *Plexauridae* are highlighted in pink and SW samples in blue. Prominent identified bands and closest GenBank relatives are listed in the accompanying table.

Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater

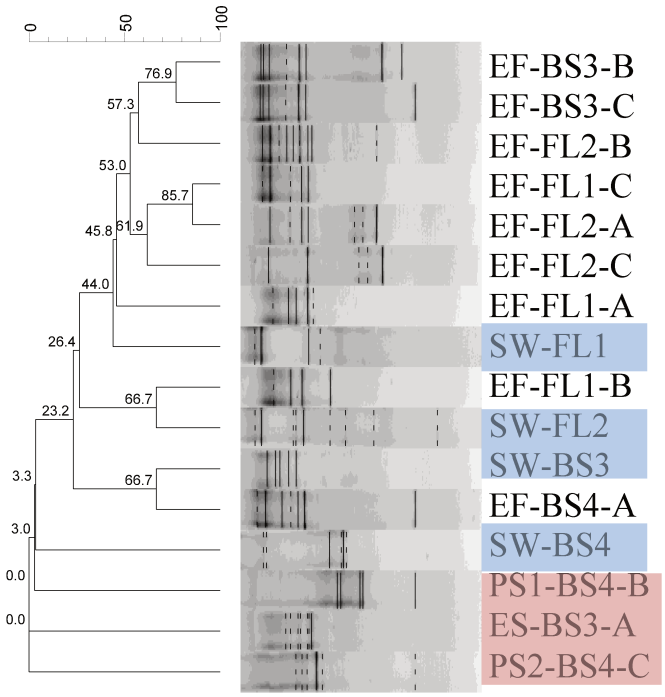


Figure 2.9 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) cluster analysis of fungal ITS denaturing gradient gel electrophoresis (DGGE) gel profiles. Non-EF *Plexauridae* are highlighted in pink and SW samples in blue.
Abbreviations: FL = Florida; BS = The Bahamas; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; A, B, C = *Plexauridae* replicates at each site; SW = seawater

2.3.4 Composition of Plexauridae Symbiodinium Communities using Symbiodinium-Specific Primers and Clone Libraries

Symbiodinium from all *Plexauridae* were identified as members of clade B1/B184 (99% sequence identity to clade B1/B184 clones or isolates).

2.3.5 Composition of Plexauridae Archaeal Communities using Archaeal-Specific Primers

Only two EF (EF-FL1-C and EF-FL2-B) contained archaeal sequences, but these sequences were not cloned and identified in this study.

2.4 Discussion

2.4.1 Composition of Bacteria in Plexauridae and Surrounding Seawater using 454-Pyrosequencing and DGGE

2.4.1.1 Geographic Heterogeneity of *E. fusca* Bacterial Communities (FL vs. BS)

The composition of EF bacterial communities (using 454-pyrosequencing) exhibited geographic variation between the FL (*Endozoicomonas*) and BS (*Mycoplasma* relatives) sites (Figure 2.3, p. 47; Figure 2.4, p. 52; Figure 2.5, p. 53). Because the 16S rDNA DGGE targeted the same hypervariable V1-V3 region as the 454-pyrosequencing, the sequencing results were directly comparable enabling cross-validation of the geographic heterogeneity observed between the EF-FL and EF-BS octocoral. The 16S rDNA DGGE also confirmed the dominance of the *Endozoicomonas*-related sequences in EF-FL samples (Figure 2.6, p. 55).

Geographic heterogeneity has been previously observed in corals[41,42,45,48,67,87,92,95,97,176,221] and other marine invertebrates.[62] Very similar to this study, Kellogg and colleagues (2009)[41] found that clone libraries of the scleractinian coral, *Lophelia pertusa*, from two locations in the Gulf of Mexico were dominated by *Gammaproteobacteria* related to sulfur-oxidizing gill symbionts of bivalves at one geographic location and *Mycoplasma* at the other.

The observed geographic heterogeneity may be due to a diseased state in the Floridian or Bahamian corals,[45,73,92,95] but there were no obvious signs of disease in collected corals. Thus, the

shift in community structure may be an adaptive response to local biotic and abiotic factors (*e.g.* temperature, anthropogenic influences, light availability, UV irradiance, pressure, salinity, pH, and/or plankton levels), which drive changes in community structure.[41,42,45,64,67,87,90,221] Therefore, it is not necessarily the location that is responsible for the observed bacterial community differences, but rather, the response of the community to the environmental factors that co-vary with the locations, as has been observed in many other corals (Table 2.4). More sampling, both geographically and temporally, is needed to ascertain if the observed geographic variation is due to a disease state at one site, or if all EF are healthy, and the variation is simply caused by local biotic and abiotic factors at each location.

Table 2.4 Bacterial heterogeneity patterns observed in other corals. Similar patterns were observed for *Eunicea fusca* at the Florida versus The Bahamas locations.

Type of Culture-Independent Study (Year)	Marine Invertebrate(s) Species (type, zooxanthellate?)	Location, No. of Sites, Replicates (Depth)	Reported Type of Heterogeneity Caused by Co-Varying Abiotic or Biotic Factors	Rationale for Heterogeneity	Ref. No.
Clone library (2004)	*Lophelia pertusa* (scleractinian coral, azooxanthellate)	Gulf of Mexico, two sites, triplicate (310-474 m)	*Geographic*: γ-proteobacteria related to sulfur-oxidizing gill symbionts of seep clam dominant at one site; *Mycoplasma* at other; both groups stable symbionts in all coral	Environmental factors: temperature, seep proximity, nutrition	41
Clone library, DGGE, T-RFLP (2007-08)	*Acropora millepora*, *A. tenuis*, & *A. valida* (scleractinian corals, zooxanthellate)	Great Barrier Reef (GBR), Australia, two sites, triplicate (2-4 m)	*Geographic*: Dominant members of community differ between locations for closely-related (same genus) corals: Magnetic Island dominated by *Endozoicomonas* & *Marinobacter*; Orpheus Island dominated by *Achromobacter* & *Brevundimonas*)	Environmental factors: nutrients, light, temperature, anthropogenic influence by coastal pollution	42
Pyrosequencing & DGGE (2009)	*Montastraea faveolata* & *Porites astreoides* (scleractinian corals, zooxanthellate)	Florida and Caribbean, four sites, replicates (5-15 m)	*Geographic & Taxonomic*: *P. asteroides* dominated by *Endozoicomonas*, but dominance decreases offshore to coast; *M. faveolata* dominated by *Burkholderiales*, but dominance decreases offshore to coast	Coral host species, environmental factors: location, depth, temp., proximity to sessile organisms, anthropogenic stress	67
DGGE (2005)	*Stylophora pistillata* & *Acroporia hyacinthus* (scleractinian corals, zooxanthellate)	GBR, Australia, three sites, replicates (3-6 m)	*Geographic, Taxonomic, & Health-State*: Two coral species had different bacterial communities that also differed at three sites; healthy samples dominated by *Endozoicomonas*, but different average contributions by site; diseased samples dominated by *Vibrios* and Rhizobiaceae bacteria	Localized environmental factors, coral host species	87
Clone library (2004)	*Plumarella superba* & *Cryogorgia koolsae* (octocorals, azooxanthellate)	Aleutian Islands, Alaska, four sites, duplicate (86-96 m, 110-138 m)	*Depth*: γ-proteobacteria dominated at two deeper sites; *Mycoplasma* dominated at two shallower sites; these groups were stable symbionts in all samples	Environmental factors at sites (depth, pressure, nutrition)	176
Clone library (2007-08)	*Paramuricea clavata* (octocoral, zooxanthellate)	Northwest Mediterranean, three sites, triplicate (20 m)	*Temporal*: Winter library dominated by *Endozoicomonas*-relatives; summer library dominated by *Firmicutes* relative	Environmental factors (temperature)	92, 180
Clone library, DGGE, FISH (2009)	*Acropora muricata* (scleractinian coral, zooxanthellate)	GBR, Australia, one site, replicates	*Health-State*: *Endozoicomonas*-relatives, *Sphingobacterium*, *Hydrogenophaga*, and *Roseobacter* dominate healthy corals; *Clostridium*, *Flavobacteria*, *Nocardioides*, and *Stenotrophomonas* dominate antibiotic-treated corals	Healthy vs. diseased state of coral	97
Clone library, DGGE (2000-03)	*Acropora millepora*, (scleractinian, zooxanthellate)	GBR, Australia, one site, replicates over a few years (1.5-3 m)	*Health-State*: *Endozoicomonas* dominate healthy corals; *Vibrios*, *Stenotrophomonas*, *Brevundimonas*, *Serratia* dominate bleached corals	Healthy vs. diseased state of coral	95

Abbreviations: DGGE = denaturing gradient gel electrophoresis; T-RFLP = terminal restriction fragment length polymorphism; FISH = fluorescent in situ hybridization; Ref. No. = reference number

2.4.1.2 Stable Bacterial Associates of *E. fusca* Across Geographic Locations

Even though EF-FL and EF-BS were dominated by different groups of bacteria, all samples contained certain stable groups of bacteria. This geographic heterogeneity, yet stable symbiosis with a few bacterial associates, has been previously reported,[41,42,67,176] and these bacterial groups likely play critical roles in the coral holobiont. The stable EF bacteria included members of the genera *Endozoicomonas* and *Mycoplasma* relatives, as well as other bacteria in the order *Oceanospirillales* (*Oceanospirillium*, 'unclassified' *Oceanospirillaceae*, and 'unclassified' *Hahellaceae*). In particular, 'unclassified' members of the family *Hahellaceae* (94% 16S rDNA identity to sulfur-oxidizing gill symbionts of bivalves and 92-94% related to *Endozoicomonas* spp.) were found in *all* EF samples (except EF-BS3-C likely due to a low number of produced pyrosequences, Table 2.2, p. 45) and *not* in the other non-EF *Plexauridae* or the surrounding seawater. Moreover, a previous study[178] that investigated the culture-independent bacterial community of *E. fusca* using a small clone library contained the same *Hahellaceae* relative (99% 16S rDNA sequence identity to these aforementioned EF-associates). This suggests that these *Hahellaceae* are important members of the EF bacterial community, not only across geographic locations, but also across time, as this previous study was carried out in 2005, four years prior to the current study. Thus, these bacteria are likely true EF-associates and should be further investigated for their biosynthetic potential to produce EF diterpenes using metagenomic techniques, or if cultured, using fermentation and genomic mining methods.

In addition to these three groups of bacteria, there were other groups (*e.g.* *Oceanospirillium* and *Labrenzia*) that were shared between Floridian and Bahamian EF (Figure 2.10, orange "Both" box), although these groups were not necessarily found in all replicates at all sites and are likely not required EF bacterial associates. However, these OTUs may have been present in all replicates, but their absence in some libraries may be due to a limit of sequencing depth in individual samples,[222] thus demonstrating the importance of replicates.

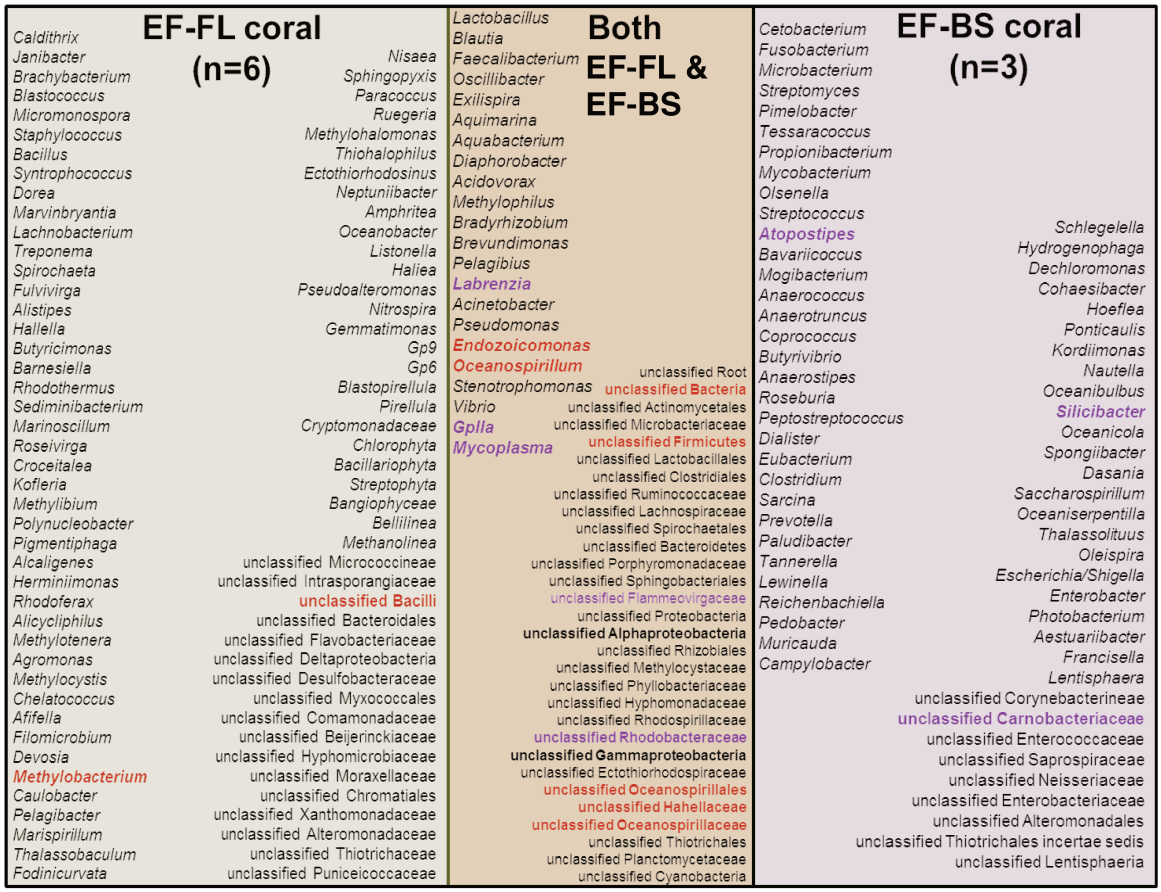


Figure 2.10 Genera of bacteria (using 454-pyrosequencing) present in Floridian *E. fusca* (EF) only (yellow box), in Bahamian *E. fusca* only (pink box), and shared between the EF from both sites (orange box). Sequences were not necessarily found in *all* EF at the respective locations. Bolded sequences: ≥50 sequences in the libraries; orange text: ≥50 sequences in EF-FL; fuchsia text: ≥50 sequences in EF-BS.
Abbreviations: FL = Florida; BS = The Bahamas; EF = *Eunicea fusca*

2.4.1.3 Comparison of Bacterial Communities Based on *Plexauridae* Host Species at Similar Geographic Locations (The Bahamas Only)

All Bahamian *Plexauridae* were dominated by *Mycoplasma* or *Spiroplasma* relatives, suggesting that these bacteria are fulfilling certain roles for these Bahamian octocorals (Figure 2.3, p. 47). This trend, of corals in the same family at the same geographic location sharing similar bacterial groups, has been previously observed.[42,61] The complete absence of *Mycoplasma* relatives in PS1-BS4-B is likely due to the abundance of the closely-related *Spiroplasma* relative. These bacteria likely carry out similar roles to the *Mycoplasma* relative and/or may actively exclude the *Mycoplasma* relative. Other groups were shared (*e.g. Endozoicomonas*) between BS *Plexauridae*, although not necessarily in *all* replicates (Figure 2.11, dark purple "Both" box). These shared groups may also carry out important functions for the BS *Plexauridae* octocorals, but may not be necessary for holobiont functioning.

There were also groups not shared between the EF-BS and non-EF-BS octocorals, providing evidence for a species-specific relationship of EF with certain groups of bacteria (*e.g. Atopostipes*, *Labrenzia*, 'unclassified' *Hahellaceae*, *Carnobacteriaceae*, and *Flammeovirgaceae*) in The Bahamas (Figure 2.11).

Endozoicomonas and *Mycoplasma* relatives were detected in the microbiomes of both Bahamian EF and non-EF *Plexauridae*. Thus, these genera may play important roles in *Plexauridae* in general and may not be just EF-specific associates. Moreover, previous studies of octocoral-bacterial communities have revealed an abundance of *Endozoicomonas* and *Mycoplasma* relatives (Table 2.5), suggesting that these groups are important in other octocoral species from diverse geographic locations. However, it is likely that each coral species contains unique, species-specific phylotypes of these bacterial groups.[39]

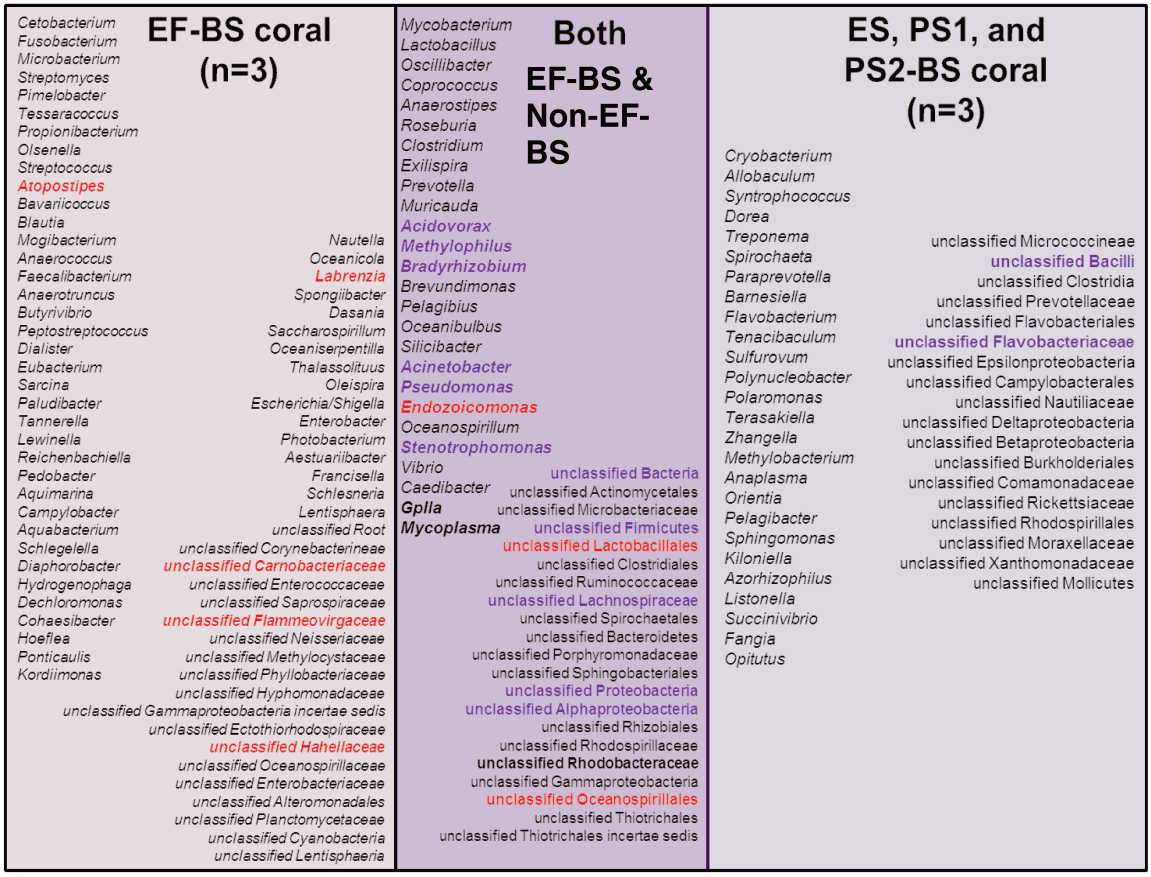


Figure 2.11 Genera of bacteria (using 454-pyrosequencing) present in EF-BS only (pink box), in ES, PS1, and PS2-BS only (purple box) and shared between the *Plexauridae* (fuchsia box). Sequences were not necessarily found in *all* samples. Bolded sequences: ≥50 sequences; red text: ≥50 sequences in EF-BS; purple text: ≥50 sequences ES, PS1, and PS2-BS.
Abbreviations: FL = Florida; BS = The Bahamas; EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2

Table 2.5 Summary of previous octocoral bacterial diversity studies containing *Endozoicomonas* and *Mycoplasma* relatives.

Octocoral(s)	Type of Culture-Independent Study	Location (Depth), Date	*Endozoicomonas* and/or *Mycoplasma* relatives	Ref. No.
Leptogorgia minimata, *Swiftia exertia*, & *Iciligorgia schrammi* (azooxanthellate)	Cultured library, FISH	Florida (45 m), 2005	Cultured *Oceanospirillales* and *Mycoplasma*	47
Eunicea fusca (zooxanthellate)	Clone library	Florida (10 m), 2005	Two *Endozoicomonas*-related sequences	178
Bamboo coral (family Isididae) (azooxanthellate)	Clone library	Gulf of Alaska, 2005	Dominated by *Mycoplasma*	32
Muricea elongata (zooxanthellate)	Clone library	Florida, 2006	Dominated by *Mycoplasma*	348
Gorgonia ventalina (zooxanthellate)	Clone library	Curacao, Netherlands Antilles, 2004	Abundant *Endozoicomonas* sequences in healthy coral	177
Paramuricea clavata (zooxanthellate)	Clone library	NW Mediterranean coast (20 m), 2007-08	Winter library, 100% of clones *Endozoicomonas* sequences; summer library, 86% of clones "Firmicutes"	92
Gorgonia ventalina (zooxanthellate)	Pyrosequencing	Bocas del Toro, Panama (1.5-5.5 m), 2008	*Endozoicomonas*-related sequences most abundant	61
Paragorgea arborea, *Plumarella superba*, & *Cryogorgia koolsae* (azooxanthellate)	Clone library	Aleutian Islands, Alaska (86-96 m), 2004	*Mycoplasma* dominant at two sites; γ-proteobacteria dominant at other two sites	176
Two *Sarcophyton* spp. (zooxanthellate)	Pyrosequencing	Red Sea Coast (8-19 m), 2009	*Endozoicomonas* sequences in corals	66
Alcyonium antarcticum	DGGE, FISH, clone library, cultured library	McMurdo Sound, Antarctica (18-25 m), 2002	All corals, all sites dominated by *Endozoicomonas*-relative	94

Abbreviations: DGGE = denaturing gradient gel electrophoresis; FISH = fluorescent in situ hybridization; Ref. No. = reference number

2.4.1.4 Comparison of *Plexauridae* 454-Pyrosequencing Data to Previous Pyrosequencing Studies

Table 2.6 compares previous bacterial 454-pyrosequencing studies carried out on marine invertebrates to the current study. Differences observed between the 454-pyrosequencing studies are due to differences in PCR primer selection, 16S rDNA hypervariable regions sequenced, sequencing depth, coral host species, geographic locations, and/or environmental factors between the studies.

Because different hypervariable regions were examined across the studies, the sequences are not directly comparable; however, the taxonomic classification of the sequences can be compared. The number of phyla (22) recovered from the *Plexauridae* was similar to a number reported for other coral species (21).[66] All *Plexauridae* in this study contained the phylum *Proteobacteria*, and many were dominated by the class *Gammaproteobacteria*, similar to previous coral studies (Table 2.6).[61,66,67] Some of the ubiquitous phyla among the octocorals in this study (*i.e. Firmicutes*, *Cyanobacteria*, *Tenericutes*, *Proteobacteria*, and *Bacteroidetes*) have also been reported to be ubiquitous in previous coral studies (Table 2.6).[66,67] Finally, members of the *Endozoicomonas* genus, *Hahellaceae* family, or *Oceanospirillales* order were reported in many previous diversity studies of not only coral, but also other marine invertebrates (Table 2.6),[59,61,62,65-67,227] suggesting that these bacteria are important in a wide variety of marine invertebrates.

Table 2.6 Comparison of marine invertebrate 454-pyrosequencing data.

Marine Invertebrate(s)	Location (Depth), Date, 16S rDNA Hypervariable Region	No. of Quality-Filtered Sequences	No. of Bacterial Phyla and OTUs	Notable Groups of Microbes	Ref. No.
Eunicea fusca (EF), *Eunicea* sp. (ES), & *Plexaura* spp. (PS1 & PS2)	Florida and The Bahamas (12.5-19.0 m), June 2009, V1-V3 (average octocoral seq. length: 472 bp)	Total octocoral: 113,050 Avg. octocoral: 9,420 Total seawater: 35,621 Avg. seawater: 8,905	Total octocoral phyla: 22 Avg. octocoral phyla: 10 Avg. octocoral 97% OTUs: 139	*Proteobacteria* and *Tenericutes* dominant bacterial phyla; *Endozoicomonas* dominant in EF from FL; *Mycoplasma* dominant in EF, ES, PS2 from BS; both genera found in all EF; *Symbiodinium* clade B1/B184	This study
Pocillopora verrucosa, *Astreopora myriophthalma*, *Sarcophyton sp.1*, *Sarcophyton sp.2*, & *Stylophora pistillata*	Red Sea Coast, Saudi Arabia (8-19 m), Apr. 2009, V3-V4 (average coral seq. length: 364 bp)	Total coral: 43,579 Avg. coral: 4,842	Total coral phyla: 21 Range of coral OTUs: 119-631	*Proteobacteria* most dominant phyla; *Endozoicomonas* found in *P. verrucosa, Sarcophyton* spp., & *S. pistillata*	66
Montastraea faveolata (MF) & *Porites asteroids* (PA)	Virgin Islands, Summerland Key, Florida, Belize (5-15 m) May – Aug. 2009, V3-V4 (coral seq. length: 212-436 bp)	Total coral: 8,547 Avg. coral: 777	MF OTU avg.: 735 PA OTU avg.: 851	*Proteobacteria* most dominant phyla; *Flavobacteria* *Endozoicomonas* dominant in PA (75-99%); MF, only 9-15% *Oceanospirillales*; PA: *Symbiodinium* clade A; MF: clades A, B, and D	67
M. faveolata, *M. franksi*, *D. strigosa*, *A. palmate*, *A. cervicornis*, *P. asteroides*, & *G. ventalina* (GV)	Bocas del Toro, Panama (1.5-5.5 m), Mar. 2008, V6	Total coral: 12,987 Avg. coral: 1,855	Total coral phyla: 31 Coral OTUs range: 1,143-2,050	*Endozoicomonas*-relative dominant in GV	61
Elysia chlorotica (EC) (sea slug) & *Vaucheria litorea* (VL) (algae)	Halifax, Nova Scotia (NS) and Martha's Vineyard, MA; Aug. 2009 – June 2010, V6-V9	EC range: 5887-11,220 VL: 18,493	EC OTUs range: 199-889 VL OTUs: 1,574	*Endozoicomonas* associated with all Martha's Vineyard samples, as well as NS lab sea slugs and algae	227
Raspailia ramosa (RR) & *Stelligera stuposa* (SS) (sponges)	Ireland southwest coast (15-20 m), Nov. 2008, V1-V3 (RR avg. length = 420 bp; SS = 437 bp)	Total RR: 14,146 Total SS: 12,099 Total seawater: 12,126	RR phyla: 10 SS phyla: 8 RR 97% OTUs: 3,013 SS 97% OTUs: 570	*Proteobacteria* most dominant phylum in both sponges; *Endozoicomonas*: SS, 5%; RR, <0.1%; seawater, 1 tag	65
Hyrtios erectus (HE), *Stylissa carteri* (SC), & *Xestospongia testudinaria* (XT) (sponges)	Red Sea, Saudi Arabia (8-19 m), Apr. 2009, V5-V6 (avg. seq. length = 290-300 bp)	Total sponge and seawater: 140,000	26 phyla HE OTUs: 1020	*Proteobacteria* major phylum; SC: *Oceanospirillales* most dominant	59
Ianthella basta (IB), *Ircinia ramosa* (IR), & *Rhopaloeides odorabile* (RO) (sponges)	Great Barrier Reef, Davies Reef, Australia (15 m), Jan. 2008, V6 (seq. length: 50-60 bp)	Sponge total: 259,000	23 phyla IB 95% OTUs: 1,099 IR 95% OTUs: 1,199 RO 95% OTUs: 2,996	IB: *Endozoicomonas* relative made up 49% of all sequences	62

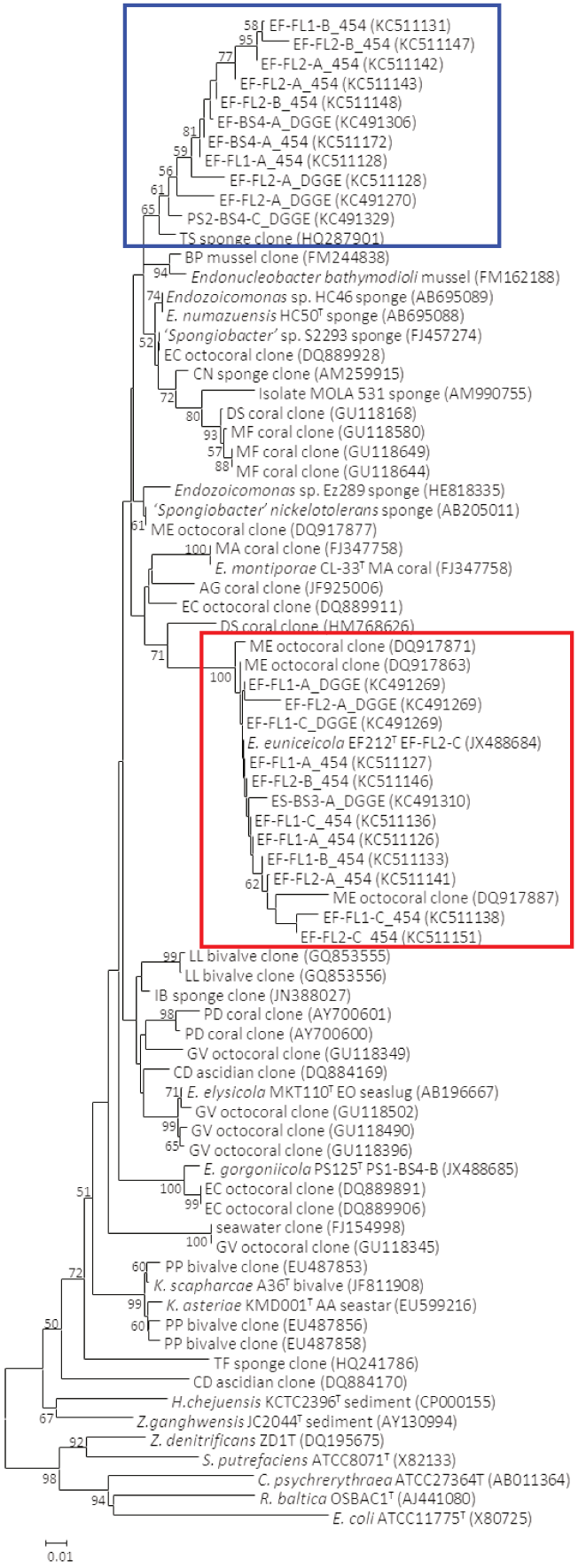
Abbreviations: V = hypervariable region of 16S rDNA; OTUs = operation taxonomic units; Ref. No. = reference number; Avg. = average

2.4.1.5 Possible Roles of *Endozoicomonas* in Octocorals and Other Marine Invertebrates

More than 50 publications describing culture-independent and -dependent microbial communities of corals and other marine invertebrates have reported *Endozoicomonas* or closely related bacteria to be dominant bacterial associates (*e.g.*[1,27,28,37,39,42,61,65-67,71,77,87-89,92,95,97,177,271-277]). These bacteria are often incorrectly referred to as the uncharacterized genus '*Spongiobacter*'[95] or other more ambiguous terms, such as Type-A Associates,[87,95] thus making it challenging to track the ubiquity of this group of bacteria across marine invertebrate species, geographic locations, and time.

Phylogenetic analysis of 16S rDNA sequences of *Endozoicomonas* and closely-related *Hahellaceae* bacteria from this study (Figure 2.12) demonstrates the relatedness of these bacteria to other clones and isolates from marine invertebrates. Phylogenetic analysis grouped the *Endozoicomonas* sequences from this study into two major clades, one with other *Plexauridae* sequences (Figure 2.12, red box) and one with sponge and bivalve sequences (Figure 2.12, blue box). Many of the other marine invertebrate isolates also grouped by host species, suggesting species-specific phylotypes may evolve for each marine invertebrate host species.[39]

Endozoicomonas bacteria have been found associated with marine invertebrates in many locations around the world and at various depths (Table 2.5, p. 67; Table 2.6, p. 69). Dinoflagellates (*Symbiodinium*) or algae are often associated with many of the marine invertebrates, and if not, the marine invertebrates are often located near hydrothermal vents or deep seeps.[278] They have been found in the gastric cavities or guts of marine invertebrates (Table 2.7) at concentrations (2.0×10^7 cells ml^{-1}) two orders of magnitude greater than in the surrounding water.[275]



Key:
Octocorals:
AG = *Alcyonium gracillimum*
EC = *Erythropodium caribaeorum*
EF = *Eunicea fusca*
ES = *Eunicea* sp.
GV = *Gorgonia ventalina*
ME= *Muricea elongata*
PS = *Plexaura* sp.
Scleractinia corals (hard corals):
DS = *Diploria strigosa*
MA = *Montipora aequituberculata*
MF = *Montastraea faveolata*
PD = *Pocillopora damicornis*
Sponges:
CN = *Chondrilla nucula*
HO = *Halichondria okadai*
IB = *Ianthella basta*
TF = *Tsitsikamma favus*
TS = *Tsitsikamma* sp.
Ascidian:
CD = *Cystodytes dellechiajei*
Sea star:
AA = *Asterias amurensis*
Gastropods:
EO = *Elysia ornata*
Bivalve:
BP = *Bathymodiolus puteoserpentis*
LL = *Loripes lacteus*
PP = *Phacoides*

454 = 454-pyrosequencing
DGGE = denaturing gradient gel electrophoresis
FL1 = Florida site 1
FL2 = Florida site 2
BS3 = The Bahamas site 3
BS4 = The Bahamas site 4
A, B, C = *Plexauridae* replicates/site

Figure 2.12 Phylogenetic relationships of 78 *Endozoicomonas* relatives based on 16S rRNA gene sequences. The evolutionary history was inferred using the Minimum Evolution method. The Neighbor-joining algorithm was used to construct the tree based on a comparison of 456 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch points; bootstrap values <50 % are not shown. The Bar length represents 0.01 substitutions per nucleotide position. Red box indicates *Plexauridae Endozoicomonas* phylotypes; blue box indicates *Plexauridae* and sponge *Endozoicomonas* phylotypes. *E. coli* ATCC 11775^{T} was used as an outgroup to root the tree.

Mouchka and colleagues[45] have reported that members of the *Oceanospirillales* order (including *Endozoicomonas*) are dominant in healthy corals, so these bacteria likely play a beneficial role in the corals. Many roles have been proposed for *Endozoicomonas* (Table 2.8) including functions related to nutrient-cycling, which is further supported by their location within the gastric cavities of corals and guts of other marine invertebrates (Table 2.7).

One of the most discussed nutritional functions is sulfur-cycling[47,279] and DMSP degradation.[67,146,170] DMSP is produced in high quantities by associated *Symbiodinium*,[160-162] and degradation products may provide nutrition,[280] act as an osmoprotectant,[281] cryoprotectant,[282] signaling molecule,[283-285] antioxidant,[168,286] and/or antimicrobial[287,288] for the coral host and other microbial associates. *Symbiodinium* are located in the gastrodermis of corals and could readily interact with these gastrodermally-located bacteria, further supporting this hypothesized role.

In addition to nutrient cycling, the *Endozoicomonas* may produce antimicrobial MNPs.[39,95,130,276,289,290] Antimicrobial activity has been reported from *Endozoicomonas* spp. against both Gram-positive and Gram-negative bacteria and yeast.[291,292]

Defining the biological roles of bacteria identified in culture-independent studies is difficult, because even closely-related microbes can have quite different phenotypes. Thus, the bacterial mode of association (symbiosis, commensalism, or parasitism) and physiological functions are often unidentified for uncultured microbes.[56,294] Fortunately, in a parallel culture-dependent study (Chapter 3) of the same *Plexauridae* samples, two novel *Endozoicomonas* spp. (*E. euniceicola* EF212^T and *E. gorgoniicola* PS125^T) were cultured that had identical 16S rDNA sequences to dominant members of this culture-independent library, allowing for further examination of the metabolism[295] and functions of these important octocoral and marine-invertebrate associates. The metabolism and roles of these bacteria will be further discussed in Chapters 4 and 5.

Table 2.7 Marine invertebrate tissue location of *Endozoicomonas* relatives.

Location, Organism(s)	Ref. No.
Gastrodermis, coral *Galaxea fascicularis*	275
Gastrodermis, corals *Acropora aspera* and *Stylophora pistillata*	349
Gut, sea slug *Elysia ornata*	350
Aggregates inside vacuoles within gill cell cytoplasm, bivalve *Acesta excavata*	331
Bacteriocytes (in trophosome), polychaetes	279, 351, 352

Abbreviation: Ref. No. = reference number

Table 2.8 Hypothesized roles of *Endozoicomonas* relatives in marine invertebrates.

Hypothesized Roles	Ref. No.
Globally-distributed cluster in benthic marine invertebrates; key role in holobiont functioning	28, 31, 42, 87, 94, 276
Adapted for different marine invertebrate hosts; use sulfur	31
Nutrient uptake; chemoautotrophs that use sulfur	47, 279
DMSP degradation, which in turn may provide UV protection, antioxidants, antimicrobials, osmoregulants, nutrition for holobiont, and chemical signals to establish bacterial communities	67, 146, 170
Polyketide or nonribosomal peptide production? (Genome of related *Hahella chejuensis* has polyketide synthases and non-ribosomal peptide synthases)	273
Exclude potential invading microbes through antimicrobial production; antimicrobials produced via quorum sensing system?	39, 95, 130, 276, 289, 290
Antimicrobial production of isolates against *E. coli, B. subtilus, Candida glabrata, Vibrio anguillarum*	291, 292
Lipolytic exoenzyme activity of isolates	353
Surfactant activity of isolates	352

Abbreviations: DMSP = dimethylsulfoniopropionate; Ref. No. = reference number

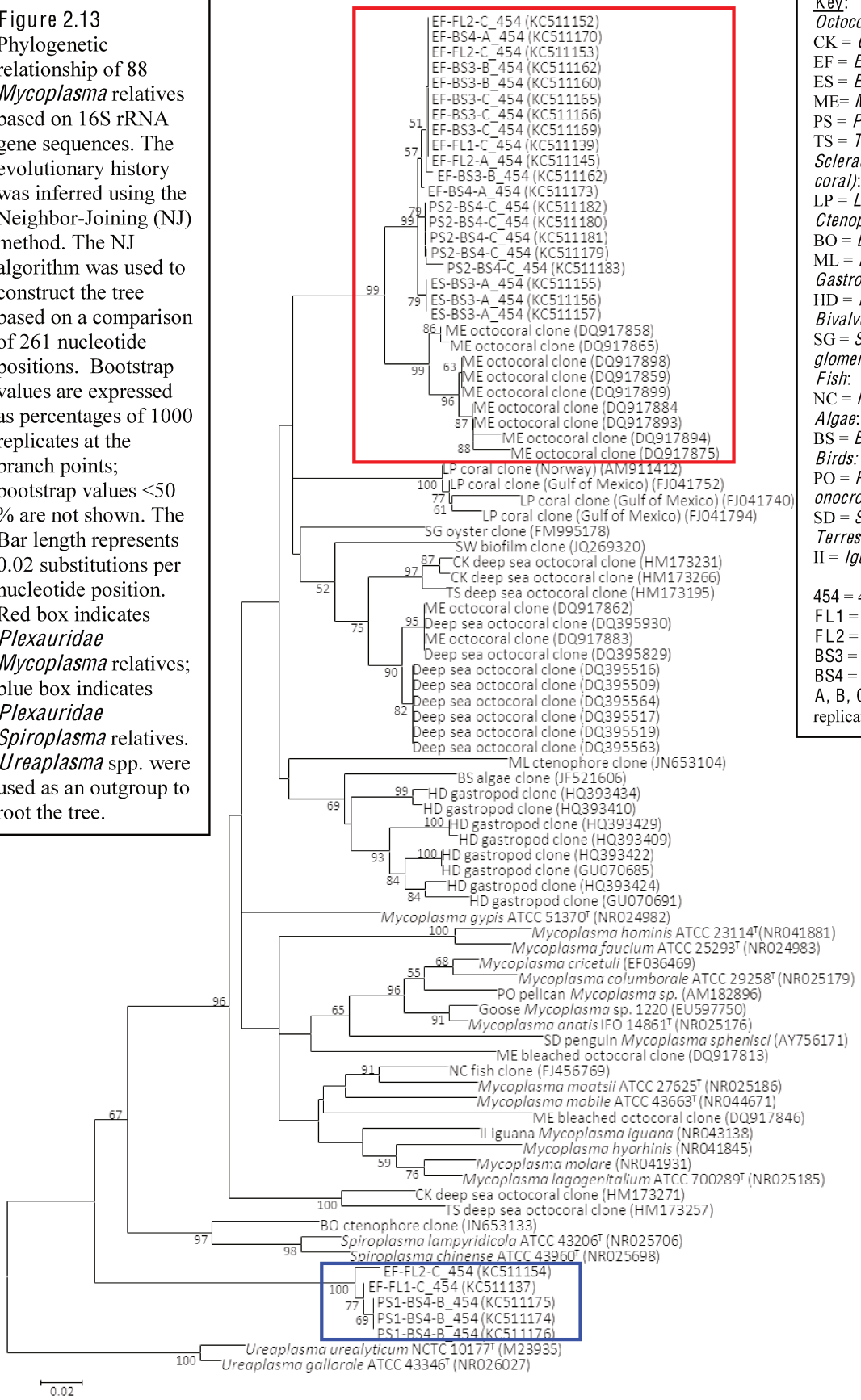
2.4.1.6 Possible Roles of *Mycoplasma* Relatives in Octocoral and Other Marine Invertebrates

The relationship of the *Mycoplasma* and *Spiroplasma* relatives detected in this study to other cultured and uncultured relatives is demonstrated in a phylogenetic tree (Figure 2.13). The *Mycoplasma* relatives from this study group closely with other octocoral *Mycoplasma* sequences from another Floridian *Plexauridae* octocoral, *Muricea elongata*.

Mycoplasma relatives have also been reported to be numerous in many corals[32,38,41,47,48,96,176] (Table 2.9) and marine invertebrates[221] from a variety of depths, with or without zooxanthellae, and are often found around areas of high hydrodynamic energy and abundant zooplankton and particulate organic matter (*e.g.* Indo-Pacific and Atlantic Margins, Mediterranean).[38]

Mycoplasma lack cell walls and have very small genomes with limited biosynthetic capabilities (*e.g.* they cannot synthesize amino and fatty acids). Therefore, most live as commensals or parasites of eukaryotes to obtain vital nutrition.[223,296,297] In corals, they have been found to be abundant in *L. pertusa* from both sides of Atlantic Ocean and are hypothesized to be chemosynthetic commensalists that provide a source of nutrition for the corals either directly, through the breakdown of hydrocarbons,[298-302] or indirectly, as the base of the food web.[38,41] The *Mycoplasma* may benefit from the association by absorbing nutrients from the sloppy feeding of the coral polyps.[48] The abundance of *Mycoplasma* relatives at the BS sites could be evidence of parasitized octocorals. However, all collected *Plexauridae* appeared to be healthy, so the *Mycoplasma* relatives are likely commensals and a normal part of the healthy, microbial consortia of the BS *Plexauridae*.

Figure 2.13 Phylogenetic relationship of 88 *Mycoplasma* relatives based on 16S rRNA gene sequences. The evolutionary history was inferred using the Neighbor-Joining (NJ) method. The NJ algorithm was used to construct the tree based on a comparison of 261 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch points; bootstrap values <50 % are not shown. The Bar length represents 0.02 substitutions per nucleotide position. Red box indicates *Plexauridae Mycoplasma* relatives; blue box indicates *Plexauridae Spiroplasma* relatives. *Ureaplasma* spp. were used as an outgroup to root the tree.



Key:
Octocorals:
CK = *Cryogorgia koolsae*
EF = *Eunicea fusca*
ES = *Eunicea* sp.
ME= *Muricea elongata*
PS = *Plexaura* sp.
TS = *Thouarella superba*
Scleractinian (hard coral):
LP = *Lophelia pertusa*
Ctenophores:
BO = *Beroe ovata*
ML = *Mnemiopsis leidyi*
Gastropod:
HD = *Haliotis diversicolor*
Bivalve:
SG = *Saccostrea glomerata*
Fish:
NC = *Notothenia coriiceps*
Algae:
BS = *Bryopsis* sp.
Birds:
PO = *Pelecanus onocrotalus*
SD = *Spheniscus demersus*
Terrestrial animals:
II = *Iguana iguana*

454 = 454-pyrosequencing
FL1 = Florida site 1
FL2 = Florida site 2
BS3 = The Bahamas site 3
BS4 = The Bahamas site 4
A, B, C = *Plexauridae* replicates/site

Table 2.9 *Mycoplasma* associated with corals.

Organism(s), Type of Study, Date, Site Details	Ref. No.
Deep sea bamboo octocoral (family Isididae), azooxanthellate dominated by *Mycoplasma*, clone library, 2005, Gulf of Alaska	32
Healthy *Muricea elongata* octocoral, zooxanthellate, dominated by *Mycoplasma*, clone library, 2006, Florida	348
Healthy *Plexaurella fusifera* octocoral, zooxanthellate, dominated by *Mycoplasma*, clone library, 2005, Bimini, The Bahamas (15 m)	96
Iciligorgia schrammi octocoral, azooxanthellate, cultured *Mycoplasma*, 2005, Florida (45 m)	47
White *Lophelia pertusa* scleractinian coral, azooxanthellate, clone library had many *Mycoplasma* sequences, 2004, Trondheimsfjord, Norway (54-264 m)	38
L. pertusa scleractinian coral, azooxanthellate, FISH showed abundance of *Candidatus* Mycoplasma corallicola in host tentacle ectoderm, 2004, Trondheimsfjord, Norway (54-264 m)	48
Lophelia pertusa scleractinian coral, azooxanthellate, clone library and DGGE dominated by *Mycoplasma* at one site, 2004, Gulf of Mexico (500 m)	41
Plumarella superba and *Cryogorgia koolsae* octocoral, azooxanthellate, clone library dominated by *Mycoplasma* at two different sites, 2004, Aleutian Islands, Alaska (86-96 m)	176

Abbreviations: DGGE = denaturing gradient gel electrophoresis; Ref. No. = reference number

2.4.1.7 Comparison between *Plexauridae* and Surrounding Seawater Bacterial Communities

When comparing *Plexauridae* diversity to the surrounding seawater, the *Plexauridae* had lower species richness, diversity, and evenness (Table 2.2, p. 45), as well as different bacterial taxonomic groups (Figure 2.3, p. 47) than the surrounding seawater samples collected at the respective locations. This trend has been observed in many marine invertebrate bacterial diversity studies,[1,27,28, 39,42,43,59,61,62,71,77,85,87,92,221,228-241] suggesting that the octocorals may select for certain groups of bacteria and can actively exclude other members from the surrounding seawater environment.

Despite the differences observed between the *Plexauridae* and seawater libraries, 38.0% of the OTUs (97% sequence similarity cut-off) in the *Plexauridae* libraries overlapped with sequences in the surrounding seawater. Moreover, 87.2% of the non-overlapping OTUs between the *Plexauridae* and seawater libraries had less than five sequences in the opposite library, suggesting that those sequences may have been found in the opposite libraries with further sequencing depth. Similar overlap has been reported in other pyrosequencing studies,[61,62] which have attributed the lack of overlap between coral and seawater libraries to a lack of sequencing depth.[242,243]

Even though 38.0% of the species-level OTUs were shared between the *Plexauridae* and the surrounding seawater, many of the overlapping groups were more abundant in the *Plexauridae* (*e.g. Endozoicomonas*), which has been observed in other corals.[61] For the abundant *Endozoicomonas* group, there were only six sequences in the surrounding seawater across all four sites. Their presence in the surrounding seawater, although minimal, may suggest horizontal acquisition (seawater to coral) by the *Plexauridae*. Horizontal transmission of bacteria has been previously demonstrated in marine invertebrates.[62,244-250] This means that low abundance bacteria found in the seawater could constitute a 'seed bank'[60,61] for the octocorals, and the octocorals may harvest beneficial microbes from this 'seed bank' and allow them to proliferate.

There were also abundant bacterial groups that were only found in *Plexauridae* (*e.g.* *Mycoplasma* relatives) and only in EF (*e.g.* 'unclassified' *Hahellaceae*), and not in the surrounding seawater. The total absence of these bacteria in the surrounding seawater may suggest vertical acquisition (from parent to larvae) of these associates. Vertical acquisition[251] has been observed in both corals and other marine invertebrates.[62,245,252-262] It may also be that the sequences were not detected in the surrounding seawater due to limited sequencing depth, and further sequencing may reveal these sequences in the seawater, as well.[62] The data presented here suggests that a combination of both horizontal and vertical transmission may be the best way to explain bacterial acquisition in the *Plexauridae* sampled in this study.[62,260]

Finally, the dominant bacteria from the seawater samples, *Gpl1a* and *Pelagibacter*, were observed in low abundances in *Plexauridae*, suggesting the octocorals or associated microbes may be able to actively exclude these groups.

2.4.1.8 Comparison of 454-Pyrosequencing Data between FL and BS Seawater and Other Seawater Bacterial Communities

The SW-FL had considerably more bacterial OTUs (average = 1,040) than the SW-BS (average = 519) samples (Table 2.2, p. 45). There were a greater number of quality-filtered sequences and a greater percentage of singletons for the SW-FL than the SW-BS samples, which may account for the increased number of OTUs for SW-FL. This increased richness may have been caused by greater pyrosequencing sampling depth for the SW-FL samples. However, the SW-FL samples may have had greater species richness due to anthropogenic affects from the coastal environment. Pommier and colleagues[60] reported a similar trend with a decrease in richness from coast to offshore.

Using a 97% sequence similarity cut-off for clustering, a large portion (~39.3%) of the seawater sequences were singletons, which is comparable to Pommier and colleagues'[60] study where 46% of all sequences were singletons. This suggests that further sampling would reveal

greater species richness, which is supported by the Choa1 richness estimate being much greater than the observed number of OTUs (Table 2.2, p. 45).

The seawater libraries from the four sites were dominated by *GpIIa* (*Prochlorococcus* and *Synechococcus*) and *Pelagibacter*. This low number of dominant taxa in the seawater has been previously observed,[54,55,263] and members of these taxa have been shown to be dominant in previous seawater culture-independent libraries.[56,62,64,71] For example, in the eastern Atlantic Ocean surface waters (≤20 m), *Pelagibacter* (SAR11 clade) constituted ~26% of the bacterial composition, and *Prochlorococcus* and *Synechococcus* made up ~10%.[64]

Similar to EF, the seawater displayed geographic heterogeneity between FL (*GpIIa*) and BS (*Pelagibacter*) sites (Figure 2.3, p. 47). In oligotrophic waters, photosynthetic cyanobacteria, including *Prochlorococcus* and *Synechococcus*, are major energy producers[264] and compete with heterotrophic bacteria (*e.g. Pelagibacter*) for inorganic nutrients.[265] *Prochlorococcus* is the most abundant photoautotroph in tropical and sub-tropical, oligotrophic seawater.[266,267] It uses reduced nitrogen and becomes less dominant in nutrient-rich (nitrogen and phosphate) waters.[264,268] *Synechococcus* is the most dominant cyanobacteria in nutrient-rich waters.[264,265,269] *Synechococcus* was the most dominant cyanobacteria in the SW-FL (Figure 2.3, p. 47; Figure 2.6, p. 55), which may be because the near shore environment was strongly influenced by terrestrial runoff (*i.e.* anthropogenic coastal pollution and organic/inorganic sedimentation).[270] *Pelagibacter* and *Prochlorococcus* were dominant in the SW-BS (Figure 2.3, p. 47; Figure 2.6, p. 55) where there was likely less anthropogenic influence and nutrient run-off. However, these hypotheses cannot be confirmed because nutrient levels were not measured in this study.

2.4.2 Composition of Fungi in Plexauridae and Surrounding Seawater using DGGE

Analysis of fungal diversity revealed a few shared groups between many EF (Figure 2.8, p. 58). Fungi related to *Toxicocladospirum* sp., *Scedosporium* sp., and *Cylindrocarpon* sp. were detected in several of the EF samples and not in the non-EF *Plexauridae* and surrounding seawater, suggesting some level of species-specificity in the EF. A more in-depth culture-

independent analysis, such as 454-pyrosequencing, may reveal EF species-specific fungi in all samples, as DGGE usually only detects sequences in high abundances.

Most of the DGGE fungal sequences were represented by members of the phyla *Ascomycota*. Microscopic studies have shown that *Ascomycota* fungi are ubiquitously found in several genera of healthy corals.[8,11,16,115,303] Within the *Ascomycota*, most of the fungi from the present study were found within the subphylum *Pezizomycotina*, class *Sordariomycetes*, and subclass *Hypocreomycetidae* (*Scedosporium* sp., *Cylindrocarpon* sp., and *Hypocreales* sp.). The class *Sordariomycetes* was previously found to be the most abundant in a metagenomic study of hard coral, *Porites asteroides*.[36] The *Ascomycota* fungal class *Dothideomycetes* (*Toxicocladosporium* sp.) was also found in EF octocoral.

Endolithic fungi have been reported to be associated with corals.[8-17,36] While some studies suggest that fungi are parasitic to corals and endolithic algae,[12,13,115,303,304] metagenomic analysis has shown that most of the coral-associated fungal genes of *P. asteroides* encoded proteins involved in cell wall construction, glutamate, aspartate, and asparagine biosynthesis, glutamine metabolism, nitrogen metabolism, and ammonia assimilation,[36] suggesting the fungi were likely involved in holobiont nutrient metabolism and not parasitic in nature. The same is believed in the present study, supported by the fact that no pathogenic fungi (*e.g. Aspergillus* spp.[13,102,113,305]) were detected in this study.

2.4.3 Composition of Symbiodinium in Plexauridae

The association between shallow-water corals and *Symbiodinium* has been studied for decades.[2,306,307] However, geographic patterns of *Symbiodinium* clade specificity in different coral species is just beginning to be elucidated.[67,89,98,99,100,209,308-313] Similar to alterations observed in coral-associated bacterial assemblages with changing environmental conditions, *Symbiodinium* can shift or shuffle in varying environmental conditions in order to maximize the health of the coral holobiont.[310] Therefore, it is necessary to study the diversity of *Symbiodinium* in healthy

corals in order to predict unhealthy states of the holobiont in future changing environmental conditions.[89]

All *Plexauridae* (EF and non-EF) in this study were associated with clade B1/B184 *Symbiodinium*. This clade has been previously reported in several Caribbean octocorals,[314-320] including *Eunicea* spp. and *Plexaura* spp., but this is the first report of this clade specifically in *E. fusca*. The presence of the B1/B184 *Symbiodinium* clade in other Caribbean octocorals may suggest that these dinoflagellates are important members of Caribbean octocoral microbiomes.

Recent research suggests *Symbiodinium* and its photosynthetic byproduct, DMSP, are drivers of bacterial associations.[42,67,90,99,100,101,117,137,146,170,233,321] In this study, the abundance of *Endozoicomonas* and related *Hahellaceae* bacteria may be due to this relationship with the *Symbiodinium* clade B1/B184. Preliminary transmission electron microscopy (TEM) data suggests that *E. fusca* contains bacterial aggregates in the gastrodermis in close proximity to the *Symbiodinium*, suggesting a tight relationship between some bacteria and the symbiotic *Symbiodinium* B1/B184 clade (Figure 2.14, Erin McCaulay, Kerr Lab, *private communication*), but further studies (*e.g.* using FISH probes) are needed to taxonomically identify these bacteria.

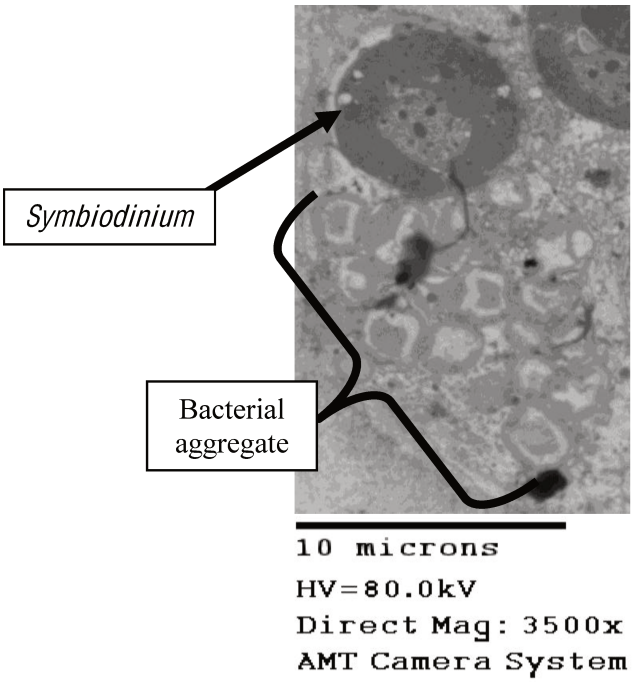


Figure 2.14 Transmission electron microscopy (3,500 times magnification) of *E. fusca* gastrodermis cross-section containing *Symbiodinium* adjacent to a bacterial aggregate. Bar length indicates 10 μm. Image courtesy of Erin McCaulay.

2.4.4 Archaeal Presence in Plexauridae

Archaea were only found in two EF samples (EF-FL1-C and EF-FL2-B) in this study using archaeal-specific primers, so they do not appear to be a major component of the *Plexauridae* microbiomes. However, more in-depth culture-independent studies, such as 454-pyrosequencing, are needed to confirm this hypothesis. Archaea have been previously reported in cold-water and tropical scleractinian corals,[36,74,75,103,322] but their functions are unknown and poorly understood.[39,74] They have not been found to be species-specific, but similar types of archaea have been found on geographically-distant and multiple species of coral; thus, a coral-specific group of archaea may exist.[36,103] The archaeal PCR amplicons were not taxonomically identified in this study as cloning was not performed, but cloning of the PCR amplicons could be done in the future to identify the groups of archaea present in the two EF samples and confirm if they are similar archaeal sequences.

2.4.5 Culture-Independent Analysis: Potential Errors and Biases

This study employed the molecular techniques of 454-pyrosequencing and DGGE in an attempt to comprehensively identify the bacterial composition of 12 *Plexauridae* octocorals and the surrounding seawater. In general, the characterization of bacterial communities using 16S rDNA PCR amplification can be negatively biased by multiple copies of the 16S rDNA gene in each bacterial genome,[323-325] variability in the amplification of the target gene,[326-328] and amplification of extracellular, environmental DNA fragments.[227] Despite these limitations, 16S rDNA amplification is a robust tool and the most commonly used molecular method for identifying bacteria in diverse environments.[227]

2.4.5.1 454-Pyrosequencing

Pyrosequencing technologies have revolutionized our ability to obtain massive amounts of bacterial diversity data in a short amount of time and at a low cost by sequencing runs performed in parallel.[337] As the mutation rates of the 16S hypervariable (V1-V9) regions are generally higher than the mean mutation rate of the 16S rRNA gene,[226,338] this approach provides

equivalent taxonomic assignment to respective full-length rRNA sequences.[339] In addition, sequencing of the V2 and V3 region allows for genus-level identification of many bacterial species[226] (V1,V2, and partial V3 were sequenced in this study). 454-pyrosequencing still requires careful evaluation of possible biases and potential errors, though.[69,339-343] Estimates of species richness may be biased by errors in pyrosequencing data[341,343] and uneven sampling efforts.[344] In this study, biases were reduced by removing failed sequences, low-quality sequence ends, tags, and primers, non-bacterial sequences, chimeras, and sequences <250 bp. This increased sample classification accuracy by reducing the effect of sequencing errors and PCR-generated chimeras.[64]

2.4.5.2 DGGE

DGGE has been routinely used in coral (*e.g.* [27-29,39,41,42,67,73,77,84,87-89,94,95,97,277]) and other marine invertebrate[233,235,241,279,329-332] microbial diversity studies. This method detects the most abundant members of the microbial community[203,333-336] and can detect major changes within and between species over space and time.[39,77,87] Even though DGGE may be unable to detect microbial ribotypes present in very low abundances, it does allow for rapid and inexpensive screening of a high number of replicate samples,[88] and can be used to cross-validate conclusions from more in-depth culture-independent methods, such as clone libraries[28,41,42,77,87,88,94,95,97,277] and 454-pyrosequencing,[67] as was demonstrated in the current study.

In this study, 16S rDNA DGGE was able to confirm the geographic heterogeneity observed in the 454-pyrosequencing bacterial communities of the *Plexauridae*, as well as confirm the abundance of *Endozoicomonas* ribotypes in the EF-FL (Figure 2.6, p. 55). However, there was a clear absence of *Mycoplasma* related sequences retrieved from DGGE that were dominant in the 454-pyrosequencing libraries. This may be explained by poor primer annealing to the *Mycoplasma* sequences. In many *Mycoplasma*, the adenine or cytosine residues at GATC sites are methylated,[223] and because this motif is part of 16S rDNA 27F primer sequence, and its reverse complement reads the same, there may be minimal binding of the 27F primer to the

sequences.[224] Thus, during the initial PCR touchdown cycle, which starts at a high melting temperature, there is likely poor primer annealing to the sequences, and as the melting temperature decreases throughout the run, other sequences are preferentially amplified. The methylation of the GATC motif may also inhibit elongation during PCR.[225] Another hypothesis for the lack of sequences is that *Mycoplasma* bands may have appeared in the DGGE profiles, but the bands may not have produced sufficient resolution in the gel to obtain a sequence. However, this latter explanation is less likely, as the bands should have appeared to be dominant in the profiles, and all dominant bands were sequenced and identified.

2.5 Conclusions

This study is one of the first in-depth, geographic investigations of octocoral microbial diversity and the first comprehensive, culture-independent analysis of the *E. fusca* microbiome. From this study, the following conclusions can be made. (1) EF has a geographically variable (FL vs. BS) bacterial community with the stable symbionts, *Endozoicomonas*, *Mycoplasma* relatives, and members of the family *Oceanospirillales* across geographic locations. These stable bacterial counterparts likely play significant roles in the functioning of the holobiont. (2) Within a geographic location (BS), EF shared some similar bacteria (*e.g. Mycoplasma* relatives) to family-related *Plexauridae* species, but also demonstrated species-specificity (*e.g. Hahellaceae* bacteria). (3) All *Plexauridae* in this study were associated with *Symbiodinium* clade B1/B184, similar to other Caribbean octocorals. (4) The fungal community showed some level of species-specificity in EF, but archaeal sequences were only retrieved from two EF. More in-depth culture-independent studies are needed to confirm these conclusions. (5) The bacterial communities of all *Plexauridae* were distinct from the surrounding seawater, suggesting that the octocorals actively select or exclude bacteria from the surrounding water.

Future culture-independent studies should investigate more *E. fusca* and a higher number of *Plexauridae* replicates of the same species across geographic and temporal scales to confirm these conclusions and further define the healthy microbial community of EF. Finally, stable EF

microbial associates should be further investigated for their biosynthetic potential to produce fuscol and related diterpenes.

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CHAPTER 3: CHARACTERIZATION OF THE CULTIVABLE MICROBIAL COMMUNITY OF *EUNICEA FUSCA* AND RELATED *PLEXAURIDAE*, AND THE DISCOVERY OF NOVEL MARINE NATURAL PRODUCTS FROM *PLEXAURIDAE*-ASSOCIATED MICROBES

3.1 Introduction

3.1.1 Cultured Octocoral-Derived Microbes as a Source of Antimicrobials

Over the past decade, the rapid emergence of antibiotic-resistant bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA)[1-3] and vancomycin-resistant *Enterococci* (VRE),[4,5] has caused an urgent need for the discovery of novel antimicrobials; this has contributed to the subsequent revival of natural products drug discovery.[6,7] Marine microbes provide an underexplored and exciting global niche to explore for novel, antimicrobial marine natural products (MNPs).[8-14] In particular, marine particle-associated microbes living on or within marine macroorganisms have been reported to produce a higher proportion of antimicrobials than free-living bacteria.[15] For example, many antibiosis and antimicrobial-resistance studies carried out with scleractinian coral-associated bacteria have shown that a high percentage (20-70%) of the total cultured bacteria are capable of producing antimicrobial substances.[16-19] The production of antimicrobials by coral-associated microbes is not surprising, as the microbes are suspected to play a major role in maintaining coral health by protecting the holobiont from invasion by other pathogenic microbes.[20-23]

Even though scleractinian coral microbial communities have been investigated for antimicrobials, octocoral microbial communities remain largely unexplored. To date, only seven culture-dependent octocoral microbial diversity studies have been carried out,[24-30] and none of these studies have explored cultured isolates for antimicrobials. Furthermore, culture-independent studies have suggested octocorals host a great diversity of unique microbes, many of which are novel species (Chapter 2).[26,27,29,30-37] Thus, these uncharacterized microbial communities provide an untapped source of microbes to explore for novel antimicrobials.

3.1.2 Cultured Eunicea fusca Microbes as a Source of Fuscol and Related Diterpenes

In addition to exploring octocoral-associated microbes as a source of novel antimicrobial MNPs, exploring these microbes as a source of known MNPs with useful biological activities is also of interest. Octocorals are known to be a prolific source of anti-inflammatory and anti-cancer

MNPs.[38-46] For example, the octocoral *Eunicea fusca* is the sole source the anti-inflammatory diterpenes, fuscol,[38] the fuscosides,[39,40] eunicol,[41] and eunicidiol.[42] However, the biosynthetic source of these compounds remains elusive. In recent years, evidence has suggested that some marine invertebrate microbial associates may contribute to the production of MNPs originally isolated from the holobiont extracts.[47-55] Therefore, it is possible that *E. fusca*-associated microbes may contribute to the biosynthesis of the reported anti-inflammatory MNPs, and the isolation of these microbes could provide a consistent yield of the desired MNPs through the use of large-scale fermentation of the producer microbe.

Previous research on the biosynthetic source of fuscol and related diterpenes has suggested biosynthesis can occur in *E. fusca*-associated *Symbiodinium* in the absence of *E. fusca* cells and is inducible by the application of plant signaling factors, such as salicylic acid, to the *Symbiodinium* preparation.[56] However, the *Symbiodinium* preparations were not free of other microbes (*e.g.* bacteria and fungi), and thus, the role of other associated microbes could not be determined.[56] A subsequent study isolated an archaeal-like farnesyldiphosphate synthase (FPPS) from an *E. fusca*-associated *Symbiodinium* preparation, and this FPPS was transcriptionally activated under conditions that led to an induction of fuscol biosynthesis.[57] It was hypothesized to have either been introduced into the *Symbiodinium* genome by horizontal gene transfer or to originate from a prokaryotic organism associated with the dinoflagellate.[57] Thus, all previous research has suggested the *Symbiodinium* symbiont may play a role in fuscol biosynthesis, but it is still uncertain whether microbes associated with the dinoflagellate may also contribute to fuscol biosynthesis.

3.1.3 Goals of this Study

In this study, the culture-dependent microbial communities of *E. fusca* and related *Plexauridae* will be characterized and then assessed as a source of MNPs. The microbial communities of these octocorals have never been thoroughly characterized, and associated

microbes may provide a source of novel antimicrobials. Additionally, *E. fusca*–associated microbes may provide a novel source of the aforementioned diterpenes.

This study will answer the following questions: (1) What is the culturable microbial community of 12 *Plexauridae* octocorals (nine *E. fusca*, one *Eunicea* sp., one *Plexaura* sp. 1, and one *Plexaura* sp. 2) from Florida and The Bahamas? (2) Are any of the cultured microbes also prominent members of the culture-independent microbial libraries (discussed in Chapter 2)? If so, this may provide insights into a biosynthetic source of fuscol. (3) Can selected cultured microbes produce novel antimicrobials? (4) Are any cultured microbes capable of fuscol biosynthesis? These key questions will be answered by carrying out a comprehensive culture-dependent study followed by analysis of selected microbes for the production of MNPs of interest.

3.2 Materials and Methods

3.2.1 Plexauridae Collection and Sample Processing

Samples (~30 g) of *Eunicea fusca* (n=9), *Eunicea* sp. (n=1), *Plexaura* sp. 1 (n=1), and *Plexaura* sp. 2 (n=1) were collected by SCUBA diving off the southeastern coast of Florida and Bimini, The Bahamas, in June of 2009 and processed as previously described (Chapter 2, section *2.2.1*, pp. 33-34). Approximately half of each *Plexauridae* sample was homogenized in sterile filtered seawater (SFSW) and subsequently separated into different sized particles (≥500 µm, ≥213 µm, ≥104 µm, ≥51 µm, <51 µm) via an adapted-particle filtration apparatus.[58] Serial dilutions (1 to 10^{-3}) of three particle sizes (<213 - ≥104 µm, <104 - ≥51 µm, and <51 µm) were prepared with SFSW, and 10 µl of the dilution was plated into individual wells in 48-well plates. Six different agar media (see Table 3.1 for all media components) were used in the 48-well plates: (1) Marine Agar (MA, 2216, BD Difco, VWR, Mississauga, ON), a high-nutrient medium targeting non-fastidious heterotrophic bacteria; (2) 1/100 R2A Agar (218263, BD Difco), a low-nutrient medium targeting slow-growing, oligotrophic bacteria; (3) BG-11[59] agar (Table 3.1 and 3.2), a low-nutrient, vitamin-supplemented medium slow-growing bacteria; (4) *E. fusca*-specific

agar, a low-nutrient medium containing *E. fusca* homogenate targeting *E. fusca*-specific bacteria (Table 3.1 and 3.3); (5) M3,[60] a medium targeting actinobacteria; (6) Modified Yeast-Malt Agar (YM), targeting fungi. All media were prepared with nystatin (50 μg ml^{-1}) (except BG-11 and YM) and cyclohexamide (50 μg ml^{-1}) (except YM) to inhibit fungal growth. Nalidixic acid (10 μg ml^{-1}) was added to M3 to inhibit fast-growing, gram-negative bacteria. Tetracycline (50 μg ml^{-1}), streptomycin sulfate (50 μg ml^{-1}), and chloramphenicol (100 μg ml^{-1}) were added to YM to prevent bacterial growth. All media components were ordered through VWR (Mississauga, ON) or Sigma-Aldrich (Oakville, ON). Plates were incubated at 22 ^{o}C for up to six months, and bacteria and fungi were purified as single colonies during this time. All isolated bacteria were grown in Marine Broth (MB 2216, BD Difco) and fungi in SYMB (Table 3.1) for two to five days and then preserved at -80 ^{o}C in 25% or 10% (v/v) glycerol, respectively.

Table 3.1 Composition of media used in this culture-dependent study.

Media Component (all g/l unless otherwise specified)	Difco MA	1/100 R2A	*E. fusca* specific	M3	YM	BG-11	SYMB
Agar	15			10	10	10	
Gellan Gum		14.85	8				
Asparagine				0.1			
Boric Acid	0.022						
$CaCl_2$	1.8						
Casamino acids		0.005					
Dextrose		0.005					
Disodium phosphate	0.008						
Ferric Citrate	0.1						
$FeSO_4$				0.01			
Glycerol				5			
K_2HPO_4		0.003		0.5			
KBr	0.08						
KCl	0.55						
Malt Extract					5		
Maltose							40
MgCl	8.8						
$MgSO_4$		0.0005		0.1			
NaCl	19.45						
NaF	0.0024						
$NaHCO_3$	0.16						
$NaSO_4^-$	3.24						
NH_4NO_3	0.0016						
Peptone	5			2			10
Proteose Peptone #3		0.005					
Sodium Propionate				4			
Sodium Pyruvate		0.003					
Sodium silicate	0.004						
Soluble Starch		0.005					
Strontium Chloride	0.034						
Yeast Extract	1	0.005			1		10
BG-11 Stock Solution (Table 3.2)						0.05 l	
DN Vitamin Mix (100X) (Table 3.2)						10 µl	
E. fusca homogenate (Table 3.3)			10				
PEI Filtered Seawater (FSW) pH 7.9		to 1 l	to 1 l	to 1 l	to 1 l	950 ml	
Instant Ocean							18
Deionized Water	to 1 l						to 1 l
Nystatin (dissolve in EtOH)	50 µg ml^{-1}	50 µg ml^{-1}	50 µg ml^{-1}	50 µg ml^{-1}			
Cyclohexamide (dissolve in DMSO)	50 µg ml^{-1}	50 µg ml^{-1}	50 µg ml^{-1}	50 µg ml^{-1}		50 µg ml^{-1}	
Nalidixic Acid (dissolve in 0.1N NaOH, H_20, filter)				10 µg ml^{-1}			
Tetracycline (dissolve in H_20, filter)					50 µg ml^{-1}		
Streptomycin Sulfate (H_20, filter)					50 µg ml^{-1}		
Chloramphenicol (add to media, autoclave)					100 µg ml^{-1}		
pH (measured)	~7.6 +/- 0.2	7.8 +/- 0.2					
Premix Instructions	Add 55.1 g	Add 0.182 g					

All media components were ordered through VWR (Mississauga, ON) or Sigma (Oakville, ON).
Abbreviations: MA = Marine Agar; YM = modified yeast-malt agar

Table 3.2 BG-11 stock solution media components.

BG-11 Additions	g/l unless specified
Nitsch's solution	
H_2SO_4 (concentrated)	0.5 ml
$MnSO_4*H_2O$	2.28
$ZnSO_4*7H_2O$	0.5
H_3BO_3	0.5
$CuSO_4*5H_2O$	0.025
$NaMoO_4*2H_2O$	0.025
$CoCl_2*6H_2O$	0.045
Distilled, deionized H_2O (diH_2O)	to 1 l
BG-11 Stock Solution	
Na_2EDTA	0.002
Citric acid	0.12
$NaNO_3$	10
$K_2HPO_4*3H_2O$	0.8
$MgSO_4*7H_2O$	1.5
$CaCl_2*2H_2O$	0.7
$Na_2CO_3*H_2O$	0.4
Ferric Ammonium Citrate	0.12
Nitcsh's solution (see above)	20 ml
$NiSO_4$ $(NH_4)2SO_4*6H_2O$ (0.1 mM solution)	5 ml
Na_2SeO_4 (0.01 mM solution)	20 ml
diH_2O	to 1 l
DN Vitamin Mix (100X stock)	mg/100 ml
Nicotinic Acid	100
PABA	10
Biotin	1
Thiamine	20
Vitamin B_{12} (cyano cobalamin)	1
Folic acid	1
i-inositol	1
Ca-pantothenate	100
BG-11 Additional Instructions:	
1. Add BG-11 Stock solution to FSW	
2. Autoclave Media	
3. Let media cool then add DN vitamin mix	
4. Pour plates	

All media components were ordered through VWR (Mississauga, ON) or Sigma-Aldrich (Oakville, ON).

Table 3.3 *E. fusca* Specific Media: Homogenate Instructions.

E. fusca Homogenate Additional Instructions:
1. Grind 10 g of *E. fusca* per liter of media in 50 ml of sterile filtered seawater (SFSW)
2. Filter through several layers of cheese cloth packed into bottom of a 60 ml syringe to remove large debris.
3. Add *E. fusca* homogenate to 1 l of SFSW and 8 g gellan gum
4. Record pH
5. Autoclave 40 min on liquid cycle

3.2.2 Taxonomic Dereplication of Microbes

Bacteria were dereplicated prior to 16S rDNA sequencing based on their protein fingerprints using Matrix-Assisted Laser Desorption/Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS). Bacterial cells grown on MA for 72 h at 30 ^{o}C were stamped onto stainless steel target plates with a sterile wooden stick, overlaid with 1.5 µl matrix (1 ml saturated solution of α-cyano-4-hydroxy-cinnamic acid (Fluka, Sigma-Aldrich) in 50% acetonitrile and 2.5% aqueous trifluoroacetic acid), and allowed to dry. Each bacterium protein profile was examined using MALDI-TOF MS (Microflex LT, Bruker Daltonics mass spectrometer, Leipzig, Germany) equipped with a 20.0-hertz (Hz) nitrogen laser (laser power 50%; up to 400 shots fired; mass range 2,000-12,000 mass-to-charge ratio [m/z]). Bacteria spectral analyses were performed by BioTyper Version 2.0 software package (Bruker Daltonics GmbH, Leipzig, Germany). For the comparison of the bacteria protein spectra, principal component analysis (PCA) was used to calculate similarity based on peak presence and intensity in each spectrum. The hierarchical clustering used a dendrogram-based algorithm to form tree-like structures from the distance of the strains (the scores of PCA) and linked them together through a linkage algorithm. *E. coli* DH15H was used as the control on each target plate (three wells per plate), and since all DH15H spectra clustered at or below 20% similarity in the constructed dendrogram, a cut-off value greater than 20% was used to define "different strains" of bacteria to be further examined by 16S rDNA analysis. Fungi were dereplicated prior to sequencing based on micromorphology using light microscopy (Leica DME, EC3 Microsystems, Heerbrugg, Switzerland).

3.2.3 Genomic DNA Extraction and PCR Amplification of Microbial Genes

For bacteria, extraction of genomic DNA (gDNA) was carried out using one of two methods. A simple dimethyl sulfoxide (DMSO) extraction was used first. For this method, 50 µl DMSO (Sigma-Aldrich) was pipetted into a sterile well in a 96-well plate, and a single bacterial colony was placed into each well with the DMSO. If DMSO was not successful in extracting the bacterial gDNA, a phenol/chloroform method was used. Briefly, 2 ml of bacterial culture (grown in MB for three days at 250 rpm and 30 ^{o}C) was pelleted, and the media supernatant was discarded. Cells were resuspended in 300 µl 50/20 Tris-ethylenediaminetetraacetic acid (EDTA) (50 mM Tris and 20 mM EDTA, pH 8.0) with 5 mg ml^{-1} lysozyme and 0.1 mg ml^{-1} RNase and incubated for 30 min at 30 ^{o}C. 50 µl of 10% SDS was added and mixed, and 85 µl of 5 M NaCl was added. 400 µl of phenol/chloroform (1:1) was added and vortexed for 30 s and then centrifuged for 10 min at 13,000 x g. The aqueous layer was retained and transferred to another tube, and 0.5 ml isopropanol was added and mixed by inversion. gDNA was pelleted at 10,000 x g, washed with 1 ml of cold 70% ethanol, allowed to dry, and resuspended in 50 µl distilled water. gDNA was extracted from fungi using a previously described phenol/chloroform extraction method.[61] All reagents were obtained through Sigma-Aldrich.

For bacteria, PCR amplification of the 16S rRNA gene was done using the universal Eubacteria 16S rRNA gene primers 27F and 1525R[62] with the following conditions: a 1X concentration of EconoTaq 2X master mix (Lucigen, Middleton, WI), 1.0 µM of each primer, 5% (v/v) DMSO and 40 ng of template DNA. PCR cycling conditions included an initial denaturing period of 3 min at 95 ^{o}C, followed by 35 cycles of 95 ^{o}C for 45 s, 54 ^{o}C for 1 min, and 72 ^{o}C for 1 min, 30 s, and a final extension of 10 min at 72 ^{o}C.

For fungi, PCR amplification of the partial 18S rRNA gene, complete ITS1 region, complete 5.8S rRNA gene, complete ITS2, and partial 28S rRNA gene was carried out using the ITS1 and ITS4 primers.[63] PCR reaction conditions were the same as above, and cycling

conditions included an initial denaturing period of 1.5 min at 95 ^{o}C, followed by 35 cycles of 95 ^{o}C for 1 min, 54.6 ^{o}C for 1.5 min, and 72 ^{o}C for 2 min, and a final extension of 5 min at 72 ^{o}C.

The presence of the correct PCR amplicons (~1500 base pairs [bp] for bacteria and ~800 bp for fungi) was verified by gel electrophoresis (120 V, 45 min, BioRad, Mississauga, ON) using 1.0% agarose gel (Agarose, Fisher Scientific) containing 0.001% ethidium bromide (Sigma-Aldrich). PCR products were visualized using a UV transilluminator (BioSpectrum®, OptiChemi HR Camera, Upland, CA).

3.2.4 Sequencing and Phylogenetic Analysis

Sequencing was performed by Eurofins MWG Operon (Huntsville, AL) or Génome Québec (Montréal, QC). Initially, the 16S 530R[64] primer was used to sequence the bacterial isolates. If any of the isolates had low sequence similarity to members in the GenBank database, then the nearly full-length 16S rDNA was sequenced using a set of six primers: 27F,[62] 530R,[64] 514F,[65] 936R,[65] 1114F,[62] and 1527R.[62] The ITS4 primer was used to sequence the fungal rDNA-ITS.

Using Contig Express (Vector NTI Advance 10.3.0, Invitrogen, Carlsbad, CA), similar sequences were grouped into OTUs (97% sequence similarity). This program was also used to assemble the nearly full-length 16S rDNA sequences. 16S rDNA and fungal rDNA-ITS sequences were further dereplicated using FastGroup[66] with a 0.01 dissimilarity value used to define different species. Unique bacterial 16S rDNA sequences (KC545231-KC545363 and JX488684-JX488685) and fungal rDNA-ITS sequences (KC545364-KC545378) were deposited in GenBank under the indicated accession numbers.

The bacterial and fungal sequences were compared against available sequences with the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov)[67] using the BLASTn algorithm in the GenBank database to determine approximate phylogenetic relatedness. For bacteria, the "16S ribosomal RNA sequences (Bacteria and Archaea)" database was used to

determine the closest, cultured phylogenetic relative, and for fungi, the "Nucleotide Collection nr/nt" with the option "Exclude Uncultured/environmental samples" was used.

Multiple sequences of related strains were aligned using BioEdit version 7.0.5.3,[68] and phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.[69] The evolutionary history of the bacteria and fungi was inferred using multiple methods: Minimum Evolution (ME),[70] Maximum-Parsimony,[71] UPGMA,[72] and Neighbor-Joining (NJ).[73] A similar topology was obtained in all phylogenetic trees generated on taxa. For the phylogenetic trees shown, the evolutionary histories of the taxa were inferred using the NJ method.[73] Bootstrap analysis was performed with 1000 resamplings,[74] and values less than 50% were collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method[75] and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset.

3.2.5 Comparison of Cultured Microbes to Culture-Independent Library

In a parallel study, the same 12 *Plexauridae* octocoral samples were investigated for their bacterial and fungal composition using the culture-independent methods, 454-pyrosequencing and denaturing gradient gel electrophoresis (DGGE) (refer to Chapter 2). In order to compare the cultured microbial isolates to the culture-independent library, a National Center for Biotechnology Information ([NCBI], Bethesda, MD) Local BLAST[67] nucleotide database containing all unique culture-independent *Plexauridae* microbial sequences was created in BioEdit version 7.0.5.3.[68] The cultured *Plexauridae* microbial sequences were searched against this local database using an Expectation (E) value of 1.0 and the Matrix BLOSUM62. Cultured sequences producing high similarity (≥97%) to members of the culture-independent library were recorded.

3.2.6 Investigating Plexauridae-Associated Bacteria for Novel Antimicrobial NPs and Fuscol

3.2.6.1 Antibiosis Study with *Endozoicomonas* spp.

The inhibitory activity of *Endozoicomonas* EF212^T and PS125^T against all unique, cultured bacterial isolates (1 x 136), as well as all cultured isolates against EF212^T and PS125^T was tested using the cross-streak method.[76] EF212^T and PS125^T were streaked down the center of large, square Petri dishes containing MA, and the other bacterial strains were simultaneously streaked perpendicular to EF212^T and PS125^T at 2.5 cm intervals (Figure 3.1).

The plates were incubated for 2-3 days at 30 ^oC, until adequate growth was observed for all strains. Antagonistic effects were indicated by the failure of the any strain to grow in the confluence zones between the *Endozoicomonas* spp. and the other bacteria. In addition, interesting phenotypic changes in the confluence zones were also noted. In order for an inhibition or phenotypic change to be considered positive, the change had to be observed in triplicate. A final inhibition test was carried out on MA by overlaying the inhibitory strains on the inhibited strains (incubated for 2-3 days at 30 ^oC) to measure the inhibition zones (Figure 3.2).

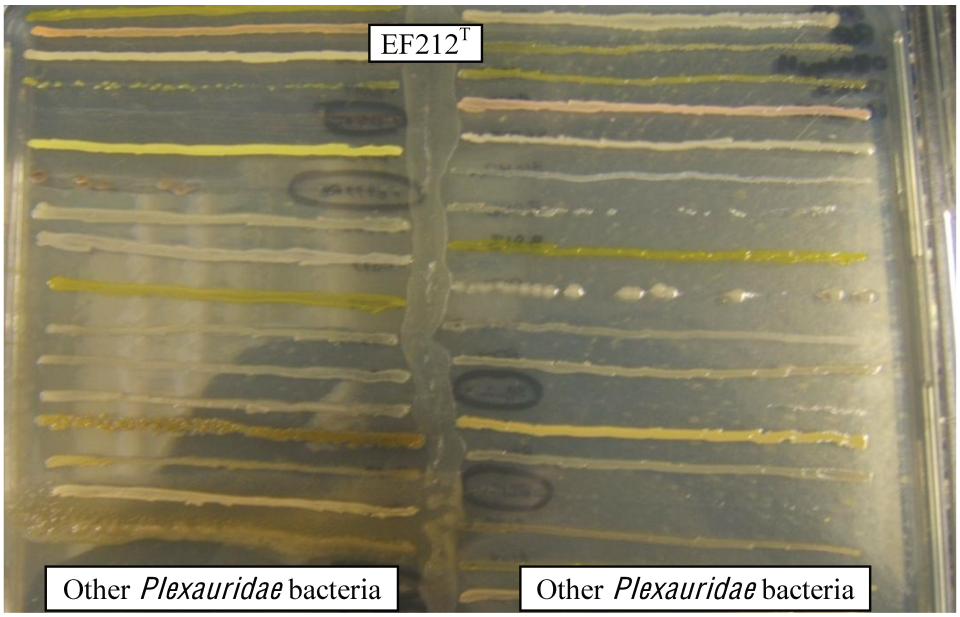


Figure 3.1 Cross-streak of *Endozoicomonas* EF212^T versus other *Plexauridae* bacterial isolates. This experiment was performed to determine potential antibiosis interactions between the isolates. Marine agar media was used, and plates were incubated for 2-3 days at 30 ^oC.

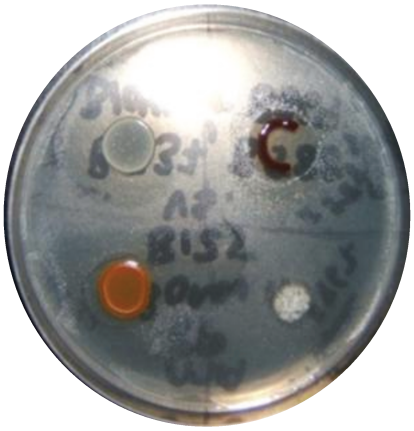


Figure 3.2 Example of final inhibition test where inhibitory bacteria were overlaid on inhibited bacteria to measure zones of inhibition. Marine agar media was used, and plates were incubated for 2-3 days at 30 ^oC.

3.2.6.2 Fermentation of *Euzebyella* sp. EF1C-B409, Compound Isolation, Structural Elucidation, and Bioactivity Screening

Euzebyella sp. EF1C-B409 was plated onto MA plates in triplicate and allowed to grow for 48 h at 30 °C. A 1.5 x 1.5 cm square of cells was scraped off each plate with a sterile cell scraper, and the cells were aseptically deposited into 10 ml of MB seed medium dispensed into 25 mm x 150 mm borosilicate glass culture tubes (VWR). Seed culture tubes were incubated at 28 °C, 200 rpm, at a 45° angle on a rotary shaker for 72 h. 1 ml of the first stage seed culture was used to inoculate seed tubes containing 10 ml of fresh medium, and this second stage seed culture was incubated under the same conditions for 24 h. Purity plates were streaked from the second stage seed culture to ensure pure cultures for the fermentation.

For the fermentation, 10 ml of seed culture was inoculated into two 4 l Fernbachs, one containing 2 l of modified Marine Broth (mMB) and the other 2 l of modified Trypticase Soy Broth (mTSB) (Table 3.4). Media blanks were included in all experiments.

Table 3.4 Fermentation media components for mMB and mTSB.

Media Component (all g/l unless otherwise specified)	Modified MB (mMB)	Modified TSB (mTSB)
Peptone	5	
Yeast Extract	1	
Ferric Citrate	0.1	
Instant Ocean	18	18
Pancreatic Digest of Casein		17
Papaic digest of soybean meal		3
Dextrose (glucose)		2.5
Distilled, deionized water	to 1 l	to 1 l
pH	7.6 +/- 0.2	7.3 +/- 0.2

All media components were ordered through VWR or Sigma-Aldrich.

Flasks were covered with a KenAG milk filter (6.5") and sterilization wrap (VWR) and secured with an autoclavable rubber band (Fisher Scientific, Toronto, ON). Fermentations were allowed to grow at 30 ^{o}C, 150 rpm for 72 h, and then were extracted with a 7/10 volume of EtOAc to fermentation and shaken at 150 rpm for 1 h. The EtOAc and aqueous layer was partitioned, and the EtOAc was retained. Extraction was repeated with an addition 200 ml EtOAc. The EtOAc extract was dried and further separated into six fractions on C_{18} Sep Pak columns (solid phase extraction, 50 mg bed weight, 1 ml column volume, Fisher Scientific) with an increasingly non-polar gradient of solvents: (1) 9:1 H_2O/MeOH; (2) 5:5 H_2O/MeOH; (3) 2:8 H_2O/MeOH; (4) 100% reagent alcohol (95% ethanol); (5) 100% acetone; (6) 5:5 DCM/MeOH. Fractions (2) through (4) were chemically screened using High Performance Liquid Chromatography (HPLC) coupled to a High-Resolution Mass Spectrometer (LC-HRMS) using a Thermo LTQ Exactive mass spectrometer with an electrospray ionization (ESI) source, an Evaporative–Light Scattering Detector (ELSD), and a photodiode array (PDA) detector. Liquid chromatography was conducted on an analytical C_{18} reverse phase column (Core Shell Kinetex C_{18}, 50 x 2.1 mm; 1.7 μm) using a standard gradient eluting from 5% acetonitrile (ACN) in water to 100% ACN with 0.1% formic acid in both mobile phases. Samples were solubilized in MeOH at a 500 μg ml^{-1} concentration. All chemicals were obtained through Fisher Scientific.

All EtOAc fractions were screened in triplicate at a concentration of 50 μg ml^{-1} against seven pathogenic microbes: methicillin-resistant *Staphylococcus aureus* (MRSA, ATCC 33591), vancomycin-resistant *Enterococci faecium* (VREF, Ef 379, Wyeth), *Staphylococcus warneri* (ATCC 17917), *Pseudomonas aeruginosa* (ATCC 14210), *Proteus vulgaris* (ATCC 12454), *Candida albicans* (ATCC 14035), and *Malassezia furfur* (ATCC 38593). Potent activity against MRSA, VRE, and *S. warneri* was observed in the C_{18} Sep Pak fraction 3 (2:8 H_2O:MeOH) for both mTSB and mMB fermentations. These fractions were further fractioned using HPLC with a Surveyor Thermo Scientific instrument and a Phenomenex Luna Phenyl-Hexyl reverse phase (RP)-HPLC Column (10 x 250 mm, 5 μm) at a flow rate of 3.0 ml min^{-1}. The purification

conditions were as follows: isocratic H_2O/MeOH 17:83 (0 – 22 min); gradient to 100% MeOH (22 – 25 min); isocratic 100% MeOH (25 – 30 min).

Fractions were re-screened for Gram-positive activity (against MRSA, VRE, *S. warneri*) and re-screened with LC-HRMS. Additional HPLC purifications were required for the isolation of the pure compound responsible for the observed Gram-positive activity. The structure of the novel antimicrobial compound (2-isononyl-5-isobutylresorcinol) was elucidated using 1-dimensional (1D) and 2-dimensional (2D) nuclear magnetic resonance (NMR) experiments (proton NMR [^{1}H], correlation spectroscopy [COSY], heteronuclear single quantum correlation [HSQC], and heteronuclear multiple bond correlation [HMBC]) as well as tandem HRMS-Mass Spectrometry (HRMS-MS) using a Thermo LTQ Orbitrap Velos mass spectrometer (50 – 100 electron volts [eV] collision energy).

NMR spectra were acquired on a Bruker Avance III 600 MHz NMR spectrometer (Bruker, Karlsruhe, Germany), and chemical shifts (δ) were reported in parts per million (ppm) and are relative to residual solvent signals: CD_3OD (^{1}H: 3.31 ppm, ^{13}C: 49.05 ppm). The signal multiplicities are reported with the abbreviations singlet (s), doublet (d), triplet (t), doublet of triplets (dt), doublet of doublets (dd), or multiplet (m).

Half maximal inhibitory concentration (IC_{50}) and minimum inhibitory concentration (MIC) values were determined for the pure compound in triplicate against the Gram-positive pathogens with 12, 1-fold decreasing concentrations (128 μg ml^{-1} to 0.0625 μg ml^{-1}). Vancomycin (MRSA and *S. warneri*) and rifampicin (VRE) were used as positive controls and DMSO as the negative control. The IC_{50} and MIC values were graphed using MasterPlex ReaderFit 2010 version 2.0.0.76 (MiraiBio, Hitachi Solutions America, Ltd., USA).

3.2.6.3 Fermentation of *Endozoicomonas* EF212^T and PS125^T and Bioactivity Screening of Extracts

The *Endozoicomonas* spp. EF212^T and PS125^T were fermented in order to determine if they produced natural products of interest (antimicrobials or fuscol). Fermentation methods were

the same as for *Euzebyella* sp. EF1C-B409, except the following media were used and prepared in 50 ml volumes: mMB, mTSB, mMB + HP20 resin, mMB + gibberellic acid (GA), mMB + methyl jasmonate (MeJA), mMB + salicin (SA), Bacteria Fermentation Media 3 (BFM3), and M9 Minimal Media (Tables 3.4 and 3.5). Plant signaling factors were previously shown to induce fuscol production,[56] and were thus used in this fermentation study. All media components were obtained through VWR, Sigma-Aldrich, or Fisher Scientific.

Fermentations were extracted as above (section 3.2.6.2, pp. 127-130) and separated into H_2O and EtOAc fractions. Antimicrobial screening was conducted as previously described except that both the H_2O and EtOAc crude fractions were screened. The EtOAc fractions from all fermentations were also screened by LC-HRMS for ions of interest (*i.e.* novel ions not found in the microbial natural products database, Antibase [Wiley-VCH, Weinheim, Germany], or fuscol-related ions, as described in the following paragraph).

In order to search for fuscol-related ions, a method was developed using XCalibur 2.0 software (Thermo Electron Corporation, San Jose, CA) to screen for the presence of ions related to fuscol in the LC-HRMS datasets. First, a fuscol standard was obtained as follows. Approximately 5 g of *E. fusca* EF-BS3-B was extracted with EtOAc and fractioned on a C_{18} Sep Pak column as previously described (section 3.2.6.2, pp. 127-130). Fractions 3 and 4 were further separated by HPLC using the same method as previously described (section 3.2.6.2, pp. 127-130). Pure fuscol was separated from the mixture, and the structure confirmed using LC-HRMS and NMR. In positive mode ESI, fuscol exhibits the characteristic ion $[M+H-H_2O]^+$ with the calculated mass ratio m/z 271.24258 at a retention time (RT) of 4.84 min. Thus, a processing method was created with XCalibur software based on these parameters (mass range: observed m/z 271.24160 +/- 5 ppm; RT: 4.84 +/- 30.00 sec) to screen all *Endozoicomonas* extracts and fractions for fuscol production.

Due to a lack of ions of interest being detected in the $EF212^T$ or $PS125^T$ fermentation extracts, no further fractionation was performed.

Table 3.5 *Endozoicomonas* spp. fermentation media used in this study.

Media Component (all g/l unless otherwise specified)	mMB+ HP20	mMB+ GA	mMB+ MeJA	mMB+ SA	BFM3	M9 Minimal Salts, 5X (premix + NaCl to 2.5% w/v)
Peptone	5	5	5	5		
Yeast Extract	1	1	1	1		
Ferric Citrate	0.1	0.1	0.1	0.1		
Instant Ocean	18	18	18	18	18	
HP20 resin	5% w/v					
GA		50 μM				
MeJA			50 μM			
SA				50 μM		
Agar					0.4	
Glycerol					12	
Soy Peptone					5	
Disodium phosphate (anhydrous)						33.9
Monosodium phosphate						15.0
Sodium Chloride						2.5
Ammonium Chloride						5.0
Distilled, deionized H_2O	to 1 l	to 1 l	to 1 l	to 1 l	to 1 l	to 1 l
pH	7.6 +/- 0.2	7.6 +/- 0.2	7.6 +/- 0.2	7.6 +/- 0.2	7.0 +/- 0.2	6.8 +/- 0.2
Additional Instructions:						Dissolve 56.4 g premix and 2.5% (w/v) NaCl, autoclave Add 200 ml autoclaved M9 minimal salts (5X) to 750 ml sterile distilled, deionized H_2O Adjust final volume to 1 l Add 20 ml filter-sterilized 20% maltose Add 2 ml filter-sterilized 1.0 M $MgSO_4$ Add 0.1 ml filter-sterilized 1.0 M $CaCl_2$

All media components were obtained through VWR, Sigma-Aldrich, or Fisher Scientific.
Abbreviations: mMB = modified Marine Broth; GA = gibberellic acid; MeJA= methyl jasmonate; SA = salicin; BFM3 = Bacteria Fermentation Media 3

3.2.6.4 Fermentation of *Labrenzia* sp. EF3B-B762, Compound Isolation, Structural Elucidation, and Bioactivity Screening

In a separate study, Nautilus Biosciences Canada Inc. (Charlottetown, PE) carried out small-scale (10 ml) fermentations of all *Plexauridae* bacterial cultures in mMB and mTSB (Table 3.4, p. 128). All EtOAc fermentation extracts (944 total) were screened for fuscol production using the previously described processing method in XCalibur (section 3.2.6.3, pp. 130-132). The presence of an ion similar to fuscol (m/z 271.242) and eluting at the same RT (4.84 min) was found in the EtOAc fermentation extract of *Labrenzia* sp. EF3B-B762 (KC545288) grown in mMB. To follow up on this lead, a 4 l scale-up fermentation was performed using mMB media, and extractions and C_{18} Sep Pak fractionations were carried out as previously described (section 3.2.6.2, pp. 127-130). C_{18} fractions (1) to (4) were analyzed via LC-HRMS (section 3.2.6.3) and were also screened for antimicrobial activity (section 3.2.6.2). C_{18} fraction 3 contained the targeted fuscol-like ion (m/z 271.242) and was further separated using HPLC with the conditions previously described (section 3.2.6.2). Pure compounds of interest were isolated, and compound structures were determined using NMR, LC-HRMS, and HRMS-MS (section 3.2.6.2).

3.3 Results

3.3.1 Taxonomic Dereplication of Microbes

From the initial dilution plating of the *Plexauridae* microbes onto the six media in 48-well plates, 1,047 bacteria and 18 fungi were isolated. Following dereplication via MALDI-TOF MS and light microscopy for bacteria and fungi, respectively, 330 bacteria and 11 fungi were found to be unique "strains." These microbes were further dereplicated based on their 16S rDNA and rDNA-ITS sequences, respectively. Using a 0.01 dissimilarity value for unique species, 137 bacteria (Table 3.6) and 11 fungi (Table 3.7) were found to be unique based on their molecular sequences. Evolutionary relationships of the unique microbes to closely-related cultured isolates are shown in phylogenetic trees based on their 16S rDNA (bacteria) and rDNA-ITS (fungi) sequences (Figures 3.3-3.7).

Table 3.6 Unique 16S rRNA gene sequences from *Plexauridae* bacteria. Green shaded "% ID" box indicates novel species and blue shaded box indicates novel genera of bacteria that had <97% or <95% 16S rDNA sequence identity, respectively, to formally characterized bacteria cultures.

Isolate Acc. No.	Seq. ID	Phylogenetic Class	Closest GenBank Relative [Acc. No.]	% ID	Seq. Length (bp)	*Plexauridae* sample from which bacteria was isolated
KC545233	*Acinetobacter* sp. EF2B-B693	*Gammaproteobacteria*	*Acinetobacter* [*calcoaceticus*] [NR_042387]	99	842	EF-FL2-B
KC545235	*Aestuariibacter* sp. ES3A-B772	*Gammaproteobacteria*	*Aestuariibacter halophilus* [NR_025721]	96	1525	ES-BS3-A
KC545307	*Alcanivorax* sp. EF2B-B769	*Gammaproteobacteria*	*Alcanivorax jadensis* [NR_025271]	98	1320	EF-FL2-B
KC545256	*Alcanivorax* sp. EF2B-B797	*Gammaproteobacteria*	*Alcanivorax hongdengensis* [NR_044499]	93	452	EF-FL2-B
KC545245	*Alcanivorax* sp. PS4B-B178	*Gammaproteobacteria*	*Alcanivorax jadensis* [NR_025271]	98	557	PS1-BS4-B
KC545236	*Alteromonadaceae* EF3B-B802	*Gammaproteobacteria*	*Alteromonas macleodii* [NR_074797]	94	1528	EF-BS3-B
KC545237	*Alteromonas* sp. EF3B-CB141	*Gammaproteobacteria*	*Alteromonas macleodii* [NR_074797]	97	476	EF-BS3-B
KC545238	*Alteromonas* sp. EF4A-B118	*Gammaproteobacteria*	*Alteromonas tagae* [NR_043977]	98	468	EF-BS4-A
KC545239	*Alteromonas* sp. PS4B-B104	*Gammaproteobacteria*	*Alteromonas macleodii* [NR_074797]	99	458	PS1-BS4-B, EF-BS3-B
KC545303	*Amphritea* sp. EF1B-B860	*Gammaproteobacteria*	*Amphritea balenae* [NR_041617]	97	1536	EF-FL1-B
KC545310	*Amphritea* sp. PS4B-B920	*Gammaproteobacteria*	*Amphritea balenae* [NR_041617]	97	1536	PS1-BS4-B
JX488684	*Endozoicomonas* sp. EF212	*Gammaproteobacteria*	*Endozoicomonas elysicola* [NR_041264]	96	1557	EF-FL2-C
JX488685	*Endozoicomonas* sp. PS125	*Gammaproteobacteria*	*Endozoicomonas elysicola* [NR_041264]	96	1556	PS1-BS4-B
KC545320	*Enterovibrio* sp. EF3B-B113	*Gammaproteobacteria*	*Enterovibrio coralii* [NR_042342]	97	490	EF-BS3-B
KC545318	*Enterovibrio* sp. EF4A-B638	*Gammaproteobacteria*	*Enterovibrio coralii* [NR_042342]	96	1535	EF-BS4-A
KC545319	*Enterovibrio* sp. EF4A-CB187	*Gammaproteobacteria*	*Enterovibrio coralii* [NR_042342]	99	1547	EF-BS4-A
KC545309	*Ferrimonas* sp. EF3B-B688	*Gammaproteobacteria*	*Ferrimonas kyonanensis* [NR_041387]	97	1549	EF-BS3-B
KC545248	*Halomonas* sp. EF1A-B807	*Gammaproteobacteria*	*Halomonas desiderata* [NR_026274]	98	471	EF-FL1-A
KC545251	*Halomonas* sp. ES3A-B328	*Gammaproteobacteria*	*Halomonas meridiana* [NR_042066]	99	471	ES-BS3-A
KC545313	*Photobacterium* sp. EF1A-B235	*Gammaproteobacteria*	*Photobacterium rosenbergii* [NR_042343]	99	460	EF-FL1-A
KC545341	*Photobacterium* sp. ES3A-B76	*Gammaproteobacteria*	*Photobacterium damselae* subsp. *damselae* [NR_040831]	100	409	ES-BS3-A
KC545342	*Photobacterium* sp. PS4B-B377	*Gammaproteobacteria*	*Photobacterium rosenbergii* [NR_042343]	98	477	PS1-BS4-B
KC545360	*Proteus* sp. PS4C-B169	*Gammaproteobacteria*	*Proteus mirabilis* [NR_074898]	99	451	PS2-BS4-C
KC545317	*Providencia* sp. PS4B-B595	*Gammaproteobacteria*	*Providencia rettgeri* [NR_042413]	99	448	PS1-BS4-B
KC545348	*Pseudoalteromonas* sp. EF1A-B164	*Gammaproteobacteria*	*Pseudoalteromonas prydzensis* [NR_044803]	96	468	EF-FL1-A
KC545234	*Pseudoalteromonas* sp. EF1B-B175	*Gammaproteobacteria*	*Pseudoalteromonas piscicida* [NR_040946]	99	557	EF-FL1-B
KC545346	*Pseudoalteromonas* sp. EF1B-CB130	*Gammaproteobacteria*	*Pseudoalteromonas piscicida* [NR_040946]	98	473	EF-FL1-B
KC545352	*Pseudoalteromonas* sp. EF3B-B256	*Gammaproteobacteria*	*Pseudoalteromonas piscicida* [NR_040946]	99	471	EF-BS3-B

K C545351	*Pseudoalteromonas* sp. EF3B-B588	*Gammaproteobacteria*	*Pseudoalteromonas piscicida* [NR_040946]	99	451	EF-BS3-B
K C545350	*Pseudoalteromonas* sp. EF3B-B799	*Gammaproteobacteria*	*Pseudoalteromonas piscicida* [NR_040946]	99	471	EF-BS3-B
K C545363	*Pseudoalteromonas* sp. EF3B-F15	*Gammaproteobacteria*	*Ferrimonas balearica* [NR_074887]	93	463	EF-BS3-B
K C545349	*Pseudoalteromonas* sp. EF4A-B537	*Gammaproteobacteria*	*Pseudoalteromonas piscicida* [NR_040946]	100	465	EF-BS4-A, ES-BS3-A, EF-BS3-B, EF-BS3-C
K C545353	*Pseudoalteromonas* sp. PS4B-B1021	*Gammaproteobacteria*	*Pseudoalteromonas phenolica* [NR_028809]	97	475	PS1-BS4-B
K C545347	*Pseudoalteromonas* sp. PS4B-B484	*Gammaproteobacteria*	*Pseudoalteromonas prydzensis* [NR_044803]	96	481	PS1-BS4-B, ES-BS3-A, EF-BS3-B, EF-BS3-C, EF-BS4-A
K C545297	*Pseudoalteromonas* sp. PS4C-CB146	*Gammaproteobacteria*	*Pseudoalteromonas prydzensis* [NR_044803]	96	464	PS2-BS4-C
K C545292	*Psychrobacter* sp. PS4C-B176	*Gammaproteobacteria*	*Psychrobacter pacificensis* [NR_027187]	99	1524	PS2-BS4-C
K C545315	*Shewanella* sp. EF3B-B536	*Gammaproteobacteria*	*Shewanella fidelis* [NR_025195]	96	336	EF-BS3-B
K C545314	*Shewanella* sp. EF3B-B556	*Gammaproteobacteria*	*Shewanella algae* [NR_028673]	96	1529	EF-BS3-B
K C545316	*Shewanella* sp. EF3C-B2	*Gammaproteobacteria*	*Shewanella algae* [NR_028673]	97	563	EF-BS3-C
K C545322	*Vibrio* sp. EF1A-B366	*Gammaproteobacteria*	*Vibrio natriegens* [NR_026124]	99	463	EF-FL1-A, EF-FL2-B, PS1-BS4-B
K C545323	*Vibrio* sp. EF1A-B655	*Gammaproteobacteria*	*Vibrio natriegens* [NR_026124]	99	440	EF-FL1-A, EF-FL1-B, EF-FL1-C
K C545324	*Vibrio* sp. EF1A-B809	*Gammaproteobacteria*	*Vibrio agarivorans* [NR_028946]	98	384	EF-FL1-A
K C545249	*Vibrio* sp. EF1A-B847	*Gammaproteobacteria*	*Vibrio fischeri* [NR_074990]	95	1535	EF-FL1-A
K C545336	*Vibrio* sp. EF1C-B615	*Gammaproteobacteria*	*Vibrio chagasii* [NR_025480]	99	1458	EF-FL1-C
K C545333	*Vibrio* sp. EF1C-B793	*Gammaproteobacteria*	*Vibrio orientalis* [NR_026127]	98	477	EF-FL1-C
K C545332	*Vibrio* sp. EF1C-CB167	*Gammaproteobacteria*	*Vibrio brasiliensis* [NR_025477]	98	1552	EF-FL1-C
K C545331	*Vibrio* sp. EF1C-CB45	*Gammaproteobacteria*	*Vibrio tubiashii* [NR_026129]	99	460	EF-FL1-C
K C545337	*Vibrio* sp. EF2B-B978	*Gammaproteobacteria*	*Vibrio sinaloensis* [NR_043858]	99	473	EF-FL2-B
K C545338	*Vibrio* sp. EF3B-B166	*Gammaproteobacteria*	*Vibrio parahaemolyticus* [NR_041838]	99	1550	EF-BS3-B
K C590063	*Vibrio* sp. EF3B-B391	*Gammaproteobacteria*	*Vibrio rumoiensis* [NR_024680]	100	398	EF-BS3-B, EF-FL2-C, EF-BS4-A
K C545327	*Vibrio* sp. EF3B-B689	*Gammaproteobacteria*	*Vibrio harveyi* [NR_043165]	98	898	EF-BS3-B
K C545330	*Vibrio* sp. EF3B-B84	*Gammaproteobacteria*	*Vibrio tubiashii* [NR_026129]	99	409	EF-BS3-B, ES-BS3-A, EF-BS4-A
K C545335	*Vibrio* sp. EF3B-CB161	*Gammaproteobacteria*	*Vibrio tubiashii* [NR_026129]	98	488	EF-BS3-B, EF-FL1-B, ES-BS3-A
K C545321	*Vibrio* sp. EF3B-CB164	*Gammaproteobacteria*	*Vibrio natriegens* [NR_026124]	98	461	EF-BS3-B
K C545326	*Vibrio* sp. EF3B-CB181	*Gammaproteobacteria*	*Vibrio rotiferianus* [NR_042081]	98	938	EF-BS3-B, EF-FL1-A, ES-BS3-A
K C545334	*Vibrio* sp. EF3B-CB182	*Gammaproteobacteria*	*Vibrio neptunius* [NR_025476]	99	1387	EF-BS3-B
K C545329	*Vibrio* sp. EF3B-CB191	*Gammaproteobacteria*	*Vibrio neptunius* [NR_025476]	99	1345	EF-BS3-B
K C545312	*Vibrio* sp. EF4A-B1045	*Gammaproteobacteria*	*Vibrio tubiashii* [NR_026129]	99	485	EF-BS4-A

K C590062	*Vibrio* sp. EF4A-B59	*Gammaproteobacteria*	*Vibrio proteolyticus* [NR_026128]	98	486	EF-BS4-A
K C545344	*Vibrio* sp. EF4A-B696	*Gammaproteobacteria*	*onella pelagia* [NR_042043]	99	477	EF-BS4-A
K C545328	*Vibrio* sp. EF4A-CB189	*Gammaproteobacteria*	*Vibrio natriegens* [NR_026124]	98	492	EF-BS4-A, ES-BS3-A, PS1-BS4-B
K C545339	*Vibrio* sp. EF4A-CB199	*Gammaproteobacteria*	*Vibrio nigripulchritudo* [NR_026126]	99	440	EF-BS4-A
K C545325	*Vibrio* sp. ES3A-B421	*Gammaproteobacteria*	*Vibrio mytili* [NR_044911]	99	723	ES-BS3-A
K C545340	*Vibrio* sp. ES3A-B773	*Gammaproteobacteria*	*Vibrio hispanicus* [NR_042806]	99	483	ES-BS3-A
K C545343	*Vibrio* sp. ES3A-CB178	*Gammaproteobacteria*	*Vibrio mediterranei* [NR_029257]	99	460	ES-BS3-A
K C545345	*Vibrio* sp. PS4C-CB169	*Gammaproteobacteria*	*Listonella pelagia* [NR_042043]	99	477	PS2-BS4-C, EF-FL1-A, EF-FL1-C
K C545232	*Erythrobacter* sp. EF3C-B835	*Alphaproteobacteria*	*Erythrobacter seohaensis* [NR_025817]	99	421	EF-BS3-C
K C545311	*Kiloniellaceae* bacterium EF3B-B119	*Alphaproteobacteria*	*Kiloniella laminariae* [NR_042646]	94	1484	EF-BS3-B
K C545285	*Labrenzia* sp. EF1C-B300	*Alphaproteobacteria*	*Labrenzia alba* [NR_042378]	98	422	EF-FL1-C
K C545288	*Labrenzia* sp. EF3B-B762	*Alphaproteobacteria*	*Labrenzia alba* [NR_042378]	98	380	EF-BS3-B
K C545289	*Labrenzia* sp. ES3A-B1008	*Alphaproteobacteria*	*Labrenzia alba* [NR_042378]	97	414	ES-BS3-A
K C545290	*Labrenzia* sp. PS4B-B940	*Alphaproteobacteria*	*Labrenzia alba* [NR_042378]	99	413	PS1-BS4-B
K C545257	*Nautella* sp. EF3B-B986	*Alphaproteobacteria*	*Nautella italica* [NR_042673]	98	421	EF-BS3-B
K C545242	*Oceanibulbus* sp. EF3B-B878	*Alphaproteobacteria*	*Oceanibulbus indolifex* [NR_027563]	99	409	EF-BS3-B
K C545252	*Paracoccus* sp. EF4A-B691	*Alphaproteobacteria*	*Paracoccus alcaliphilus* [NR_042716]	96	1450	EF-BS4-A
K C545308	*Pelagibius* sp. EF2B-B188	*Alphaproteobacteria*	*Pelagibius litoralis* [NR_043785]	93	1454	EF-FL2-B
K C545265	*Pseudoruegeria* sp. EF2C-B818	*Alphaproteobacteria*	*Pseudoruegeria aquimaris* [NR_043932]	98	401	EF-FL2-C
K C545287	*Pseudovibrio* sp. PS4B-B512	*Alphaproteobacteria*	*Pseudovibrio denitrificans* [NR_029112]	100	402	PS1-BS4-B
K C545306	*Rhodospirillaceae* bacterium PS4B-B496	*Alphaproteobacteria*	*Pelagibius litoralis* [NR_043785]	93	1482	PS1-BS4-B
K C545259	*Roseovarius* sp. EF1C-B42	*Alphaproteobacteria*	*Roseovarius aestuarii* [NR_044424]	97	1465	EF-FL1-C
K C545269	*Ruegeria* sp. EF1C-B905	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	95	427	EF-FL1-C
K C545302	*Ruegeria* sp. EF1C-B951	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	97	506	EF-FL1-C
K C545266	*Ruegeria* sp. EF1C-CB31	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	97	406	EF-FL1-C
K C545267	*Ruegeria* sp. EF2B-B203	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	96	423	EF-FL2-B
K C545254	*Ruegeria* sp. EF2B-CB79	*Alphaproteobacteria*	*Ruegeria atlantica* [NR_043449]	97	997	EF-FL2-B
K C545261	*Ruegeria* sp. EF2C-B1020	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	96	410	EF-FL2-C
K C545260	*Ruegeria* sp. EF2C-B216	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	95	414	EF-FL2-C, EF-BS3-C
K C545258	*Ruegeria* sp. EF2C-B768	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	97	414	EF-FL2-C
K C545264	*Ruegeria* sp. EF2C-B774	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	96	407	EF-FL2-C
K C545262	*Ruegeria* sp. EF2C-B912	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	95	407	EF-FL2-C
K C545263	*Ruegeria* sp. EF3B-B100	*Alphaproteobacteria*	*Ruegeria mobilis* [NR_041454]	99	418	EF-BS3-B

K C545253	*Ruegeria* sp. EF3B-B312	*Alphaproteobacteria*	*Ruegeria pomeroyi* [NR_028727]	97	423	EF-BS3-B
K C545296	*Shimia* sp. EF3B-CB115	*Alphaproteobacteria*	*Shimia marina* [NR_043300]	99	1459	EF-BS3-B
K C545268	*Tateyamaria* sp. EF1C-B676	*Alphaproteobacteria*	*Tateyamaria omphalii* [NR_041255]	95	422	EF-FL1-C
K C545273	*Bacillus* sp. EF1A-B146	*Bacilli*	*Bacillus firmus* [NR_025842]	97	472	EF-FL1-A
K C545247	*Bacillus* sp. EF1A-B810	*Bacilli*	*Bacillus infantis* [NR_043267]	99	475	EF-FL1-A
K C545246	*Bacillus* sp. EF1A-B970	*Bacilli*	*Bacillus simplex* [NR_042136]	99	469	EF-FL1-A
K C545270	*Bacillus* sp. EF1B-B343	*Bacilli*	*Bacillus siralis* [NR_028709]	99	391	EF-FL1-B
K C545291	*Bacillus* sp. EF1C-CB203	*Bacilli*	*Bacillus stratosphericus* [NR_042336]	99	462	EF-FL1-C
K C545272	*Bacillus* sp. EF2C-B619	*Bacilli*	*Bacillus thuringiensis* [NR_043403]	100	456	EF-FL2-C
K C545276	*Bacillus* sp. EF2C-B777	*Bacilli*	*Bacillus algicola* [NR_029077]	98	1554	EF-FL2-C
K C545275	*Bacillus* sp. EF2C-B913	*Bacilli*	*Bacillus algicola* [NR_029077]	99	483	EF-FL2-C
K C545295	*Bacillus* sp. EF3B-B101	*Bacilli*	*Bacillus stratosphericus* [NR_042336]	97	416	EF-BS3-B
K C545240	*Bacillus* sp. EF3B-B758	*Bacilli*	*Bacillus pumilus* [NR_074977]	99	487	EF-BS3-B
K C545274	*Bacillus* sp. EF3C-B1047	*Bacilli*	*Bacillus firmus* [NR_025842]	98	489	EF-BS3-C
K C545294	*Bacillus* sp. EF4A-B1046	*Bacilli*	*Bacillus pumilus* [NR_074977]	99	485	EF-BS4-A
K C545357	*Bacillus* sp. EF4A-B288	*Bacilli*	*Bacillus aquimaris* [NR_025241]	99	1557	EF-BS4-A
K C545277	*Bacillus* sp. EF4A-B854	*Bacilli*	*Bacillus cereus* [NR_074540]	99	487	EF-BS4-A
K C545293	*Bacillus* sp. PS4B-B309	*Bacilli*	*Bacillus firmus* [NR_025842]	99	1546	PS1-BS4-B
K C545271	*Bacillus* sp. PS4C-CB147	*Bacilli*	*Bacillus infantis* [NR_043267]	98	490	PS2-BS4-C, EF-FL2-B, EF-BS4-A
K C545278	*Oceanobacillus* sp. EF2B-B194	*Bacilli*	*Oceanobacillus iheyensis* [NR_075027]	97	498	EF-FL2-B
K C545231	*Oceanobacillus* sp. EF2C-B482	*Bacilli*	*Oceanobacillus picturae* [NR_028952]	99	495	EF-FL2-C
K C545279	*Oceanobacillus* sp. EF4A-B656	*Bacilli*	*Oceanobacillus iheyensis* [NR_075027]	97	495	EF-BS4-A
K C545305	*Paenibacillus* sp. PS4B-B502	*Bacilli*	*Paenibacillus taichungensis* [NR_044428]	99	458	PS1-BS4-B
K C545250	*Staphylococcus* sp. EF1A-B1042	*Bacilli*	*Staphylococcus haemolyticus* [NR_074994]	99	485	EF-FL1-A
K C545255	*Staphylococcus* sp. EF2B-B65	*Bacilli*	*Staphylococcus saprophyticus* [NR_074999]	100	442	EF-FL2-B
K C545241	*Brachybacterium* sp. EF1C-B244	*Actinobacteria*	*Brachybacterium paraconglomeratum* [NR_025502]	99	447	EF-FL1-C
K C545243	*Brevibacterium* sp. EF2B-B191	*Actinobacteria*	*Brevibacterium casei* [NR_041996]	99	451	EF-FL2-B
K C545244	*Dietzia* sp. EF2B-B525	*Actinobacteria*	*Dietzia schimae* [NR_044482]	99	403	EF-FL2-B
K C545304	*Kocuria* sp. EF4A-B697	*Actinobacteria*	*Kocuria polaris* [NR_028924]	99	440	EF-BS4-A
K C545358	*Micrococcus* sp. EF1B-B144	*Actinobacteria*	*Micrococcus luteus* [NR_075062]	99	1516	EF-FL1-B
K C545354	*Micrococcus* sp. EF1C-CB201	*Actinobacteria*	*Micrococcus luteus* [NR_075062]	95	449	EF-FL1-C
K C545355	*Micrococcus* sp. EF2B-B193	*Actinobacteria*	*Micrococcus luteus* [NR_075062]	99	444	EF-FL2-B
K C545356	*Micrococcus* sp. PS4B-B1026	*Actinobacteria*	*Micrococcus luteus* [NR_075062]	99	453	PS1-BS4-B

K C545286	*Ornithinimicrobium* sp. EF2C-B618	*Actinobacteria*	*Ornithinimicrobium kibberense* [NR_043056]	98	1525	EF-FL2-C
K C545359	*Streptomyces* sp. EF3B-B867	*Actinobacteria*	*Streptomyces luridiscabiei* [NR_025155]	99	445	EF-BS3-B
K C545300	*Aquimarina* sp. EF1C-B1014	*Flavobacteriia*	*Aquimarina intermedia* [NR_042444]	96	1506	EF-FL1-C
K C545299	*Aquimarina* sp. PS4B-B881	*Flavobacteriia*	*Aquimarina intermedia* [NR_042444]	96	1503	PS1-BS4-B
K C545282	*Arenibacter* sp. EF4A-B386	*Flavobacteriia*	*Arenibacter echinorum* [NR_044271]	98	344	EF-BS4-A
K C545281	*Euzebyella* sp. EF1C-B409	*Flavobacteriia*	*Euzebyella saccharophila* [FN554868]	99	443	EF-FL1-C
K C545280	*Jejuia* sp. EF1C-CB108	*Flavobacteriia*	*Jejuia pallidilutea* [NR_044501]	96	1521	EF-FL1-C
K C545301	*Mesoflavibacter* sp. EF2B-B994	*Flavobacteriia*	*Mesoflavibacter zeaxanthinifaciens* [NR_041488]	99	450	EF-FL2-B
K C545362	*Mesoflavibacter* sp. EF3C-B1003	*Flavobacteriia*	*Mesoflavibacter zeaxanthinifaciens* [NR_041488]	98	476	EF-BS3-C
K C545284	*Muricauda* sp. EF1B-B200	*Flavobacteriia*	*Muricauda ruestringensis* [NR_074562]	94	1521	EF-FL1-B
K C545283	*Muricauda* sp. EF2C-B554	*Flavobacteriia*	*Muricauda ruestringensis* [NR_074562]	95	453	EF-FL2-C
K C545361	*Flammeovirga* sp. EF3B-CB140	*Cytophagia*	*Flammeovirga yaeyamensis* [NR_041395]	99	437	EF-BS3-B
K C545298	*Flammeovirgaceae* bacterium EF2C-B566	*Cytophagia*	*Roseivirga ehrenbergii* [NR_025825]	94	1503	EF-FL2-C

Abbreviations: EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; SW = Seawater; FL = Florida; BS = The Bahamas; A, B, C = Octocoral replicates at each individual site; Acc. No. = accession number; Seq. = sequence; ID = identity; bp = basepair

Table 3.7 Unique rDNA-ITS gene sequences from *Plexauridae* fungi.

Isolate Acc. No.	Seq. ID	Phylogenetic Class	Closest GenBank Relative [Acc. No.]	% ID	Seq. length (bp)	*Plexauridae* sample from which fungi was isolated
KC545376	*Sterigmatomyces* sp. PS4C-F14	*Agaricostilbomycetes*	*Sterigmatomyces halophilus* [NR_073302]	100	490	PS2-BS4-C
KC545370	*Cladosporium* sp. EF3B-F8	*Dothideomycetes*	*Cladosporium cladosporioides* [JX868638]	99	494	EF-BS3-B
KC545374	*Cladosporium* sp. PS4B-F12	*Dothideomycetes*	*Cladosporium cladosporioides* [JX868638]	99	500	PS1-BS4-B
KC545371	*Cladosporium* sp. PS4B-F9	*Dothideomycetes*	*Cladosporium* sp. A61P16 [FJ477086]	100	489	PS1-BS4-B
KC545369	*Didymellaceae* fungi EF1C-F6	*Dothideomycetes*	*Didymellaceae* sp. [JQ717314]	99	528	EF-FL1-C
KC545364, KC545373	*Ramularia* sp. EF2B-F1	*Dothideomycetes*	*Ramularia eucalypti* [EF394862]	99	466	EF-FL2-B
KC545378	*Ramularia* sp. ES3A-F18	*Dothideomycetes*	*Ramularia eucalypti* [EF394862]	99	455	ES-BS3-A
KC545366-KC545368	*Exophiala* sp. EF1A-F3	*Eurotiomycetes*	*Exophiala oligosperma* [DQ836794]	99	591	EF-FL1-A
KC545372, KC545375	*Penicillium* sp. ES3A-F10	*Eurotiomycetes*	*Penicillium corylophilum* [JQ272469]	100	510	ES-BS3-A
KC545365	*Rhodosporidium* sp. EF2C-F2	*Microbotryomycetes*	*Rhodosporidium diobovatum* [HQ670682]	99	574	EF-FL2-C
KC545377	*Sordariomycetes* fungi EF3B-F16	*Sordariomycetes*	*Sordariomycetes* sp. [JQ760828]	99	466	EF-BS3-B

Abbreviations: EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; SW = Seawater; FL = Florida; BS = The Bahamas; A, B, C = Octocoral replicates at each individual site; Acc. No. = accession number; Seq. = sequence; ID = identity; bp = basepair

(A)

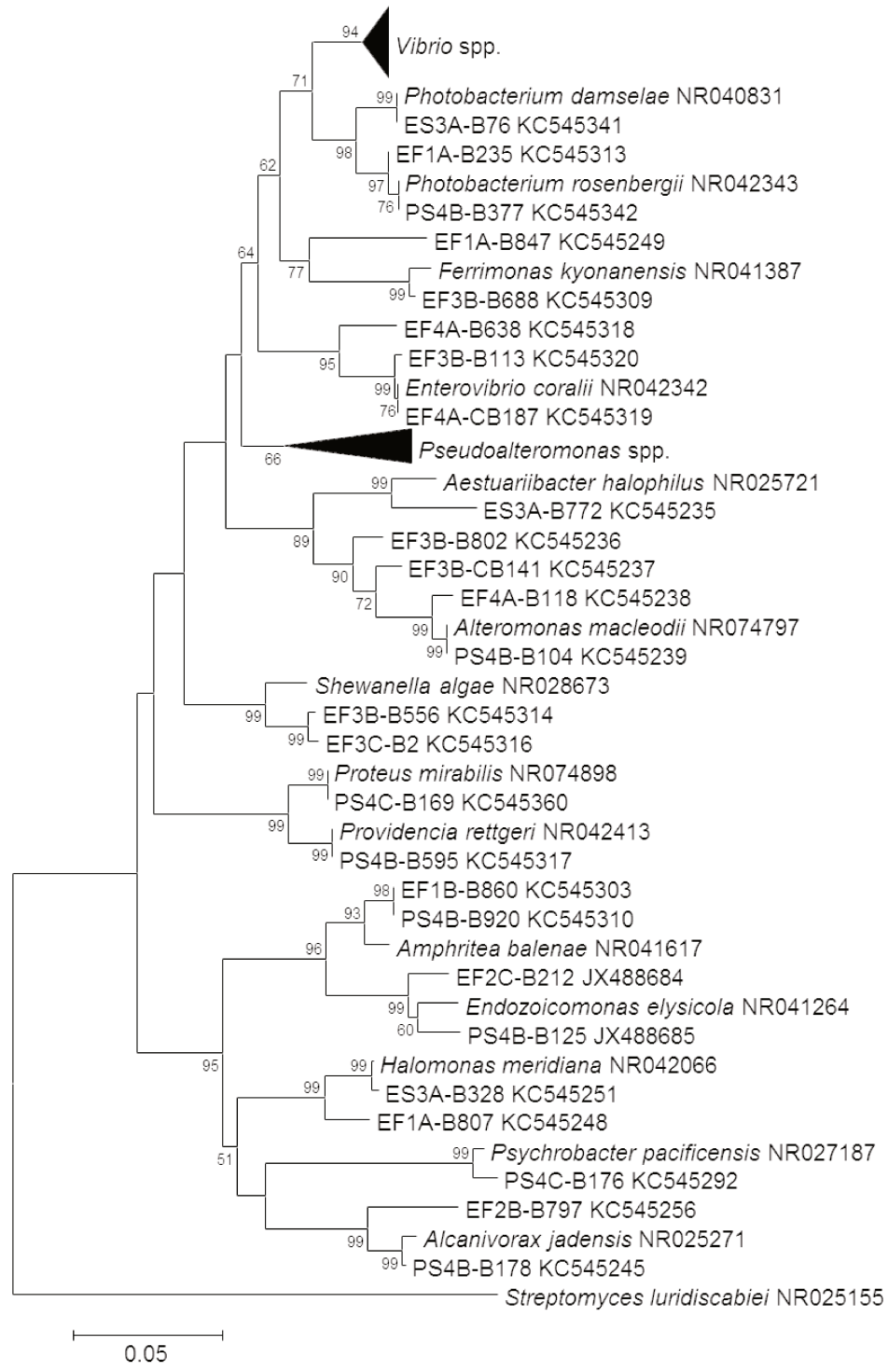
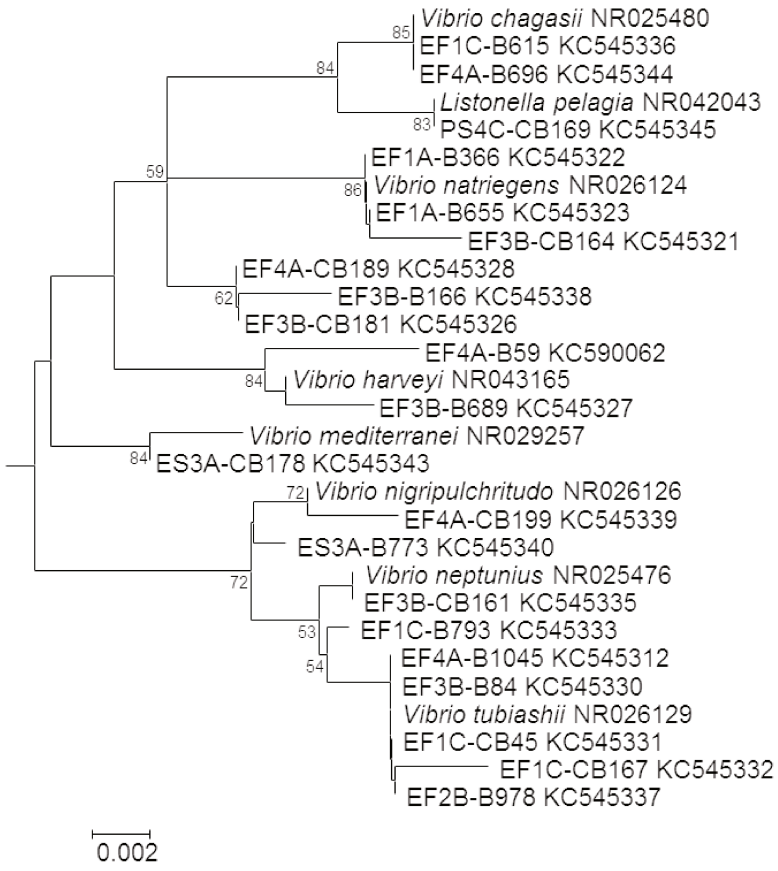


Figure 3.3 (A) Phylogenetic tree of 84 *Plexauridae Gammaproteobacteria* isolates and related type strains based on 16S rRNA gene sequences. The evolutionary history was inferred using the Neighbor Joining method. Bar length = 5 substitutions/100 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch nodes (values <50% were excluded). The phylogenetic tree was constructed based on 331 positions. NR025155 was used as the outgroup to root the tree.

(B)



(C)

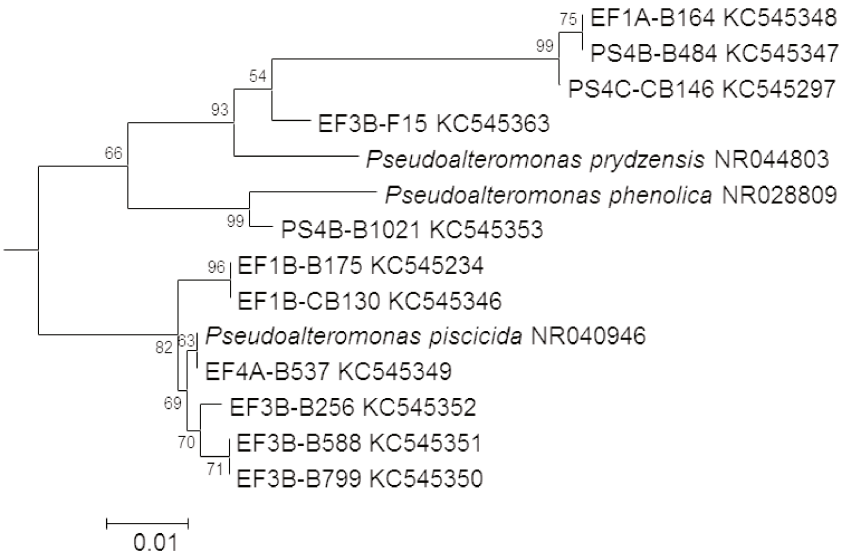


Figure 3.3 (cont.) (B) *Vibrio* spp. branch expanded (bar length = 2 substitutions/1000 nucleotide positions). (C) *Pseudoalteromonas* spp. branch expanded (bar length = 1 substitutions/100 nucleotide positions).

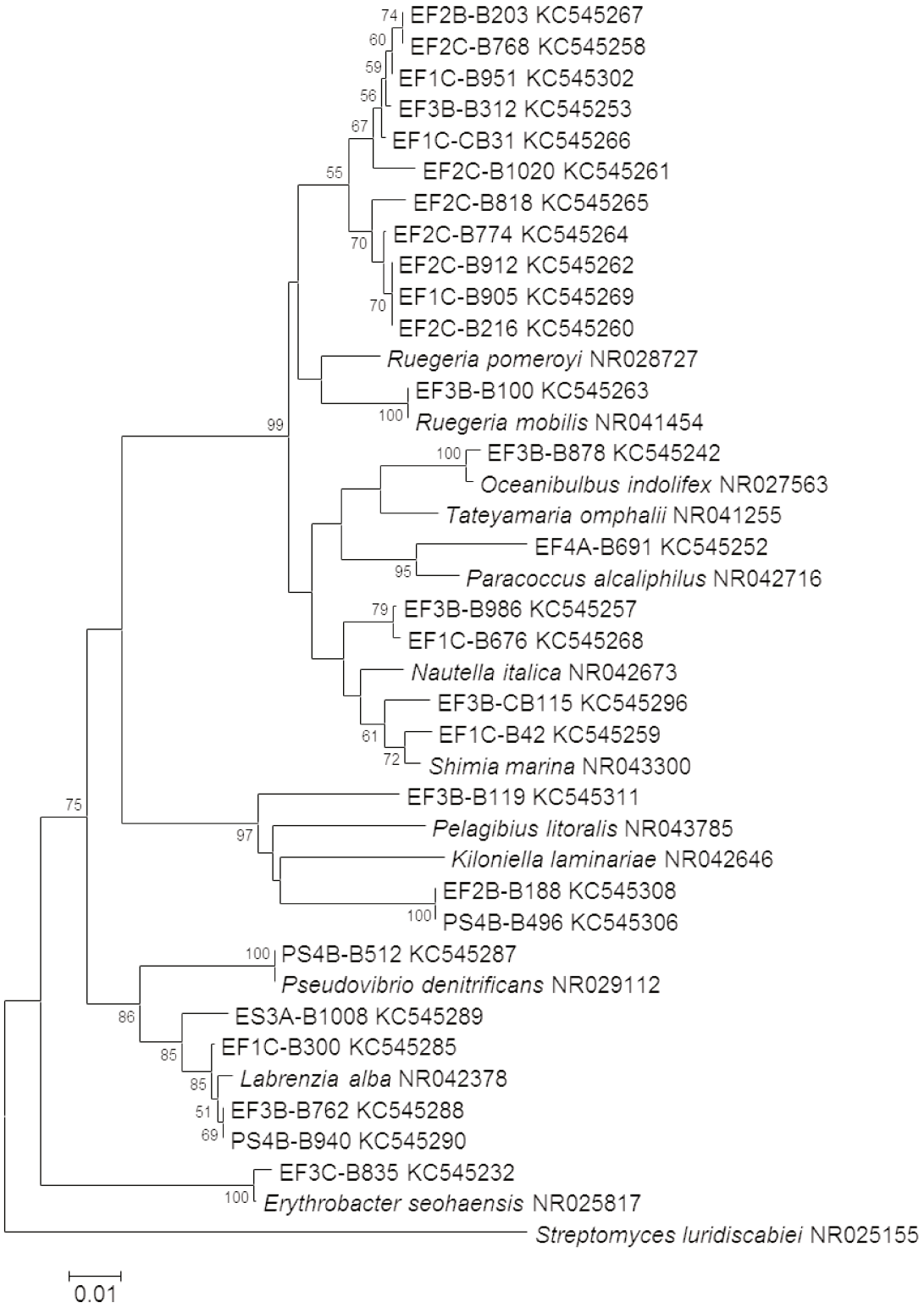


Figure 3.4 Phylogenetic tree of 40 *Plexauridae Alphaproteobacteria* isolates and related type strains based on 16S rRNA gene sequences. The evolutionary history was inferred using the Neighbor Joining method. Bar length = 1 substitution/ 100 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch nodes (values <50% were excluded). The phylogenetic tree was constructed based on 343 positions. NR025155 was used as the outgroup to root the tree.

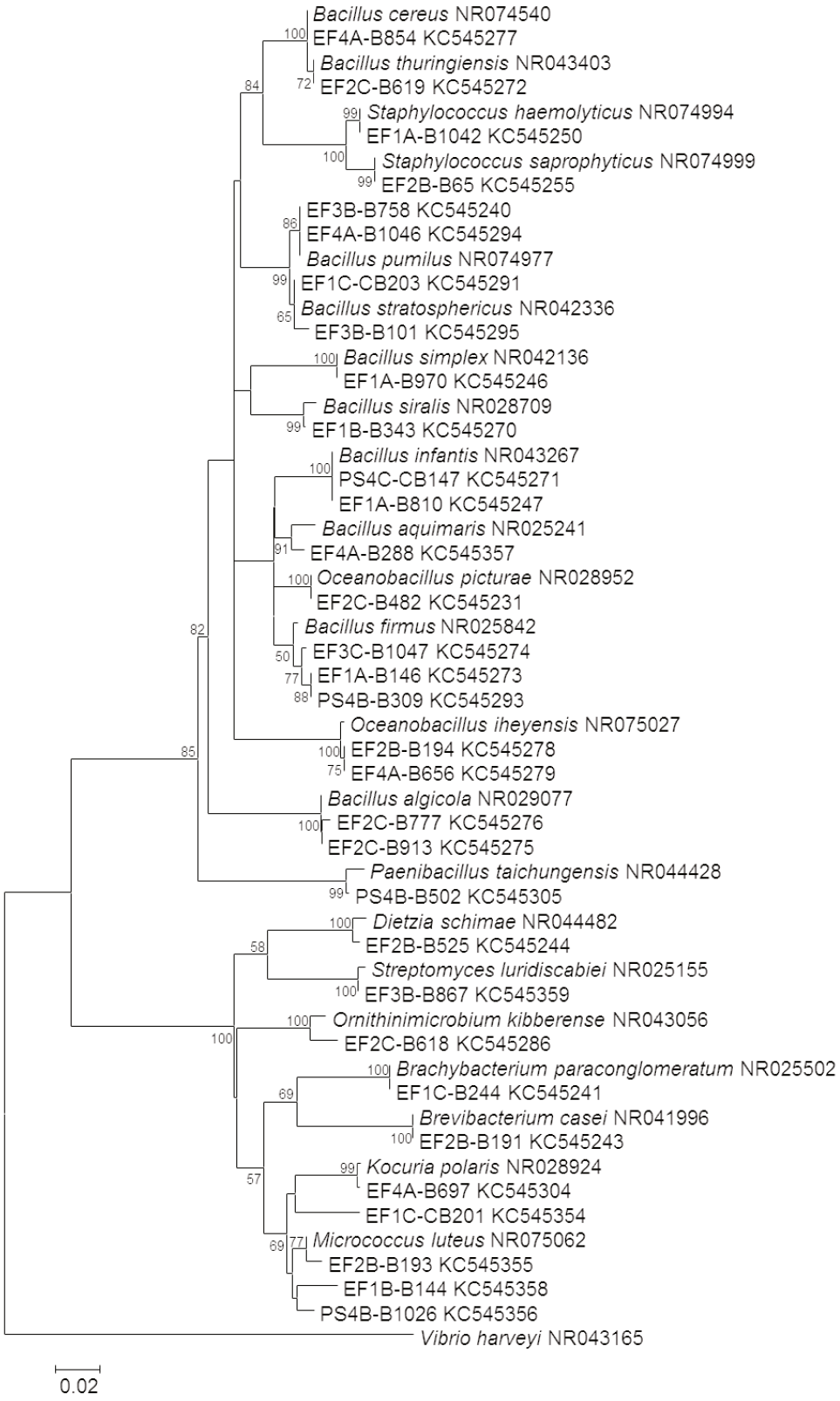


Figure 3.5 Phylogenetic tree of 55 *Plexauridae Actinobacteria* and *Bacilli* isolates and related type strains based on 16S rRNA gene sequences. The evolutionary history was inferred using the Neighbor Joining method. Bar length = 2 substitutions/ 100 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch nodes (values <50% were excluded). The phylogenetic tree was constructed based on 313 positions. NR043165 was used as the outgroup to root the tree.

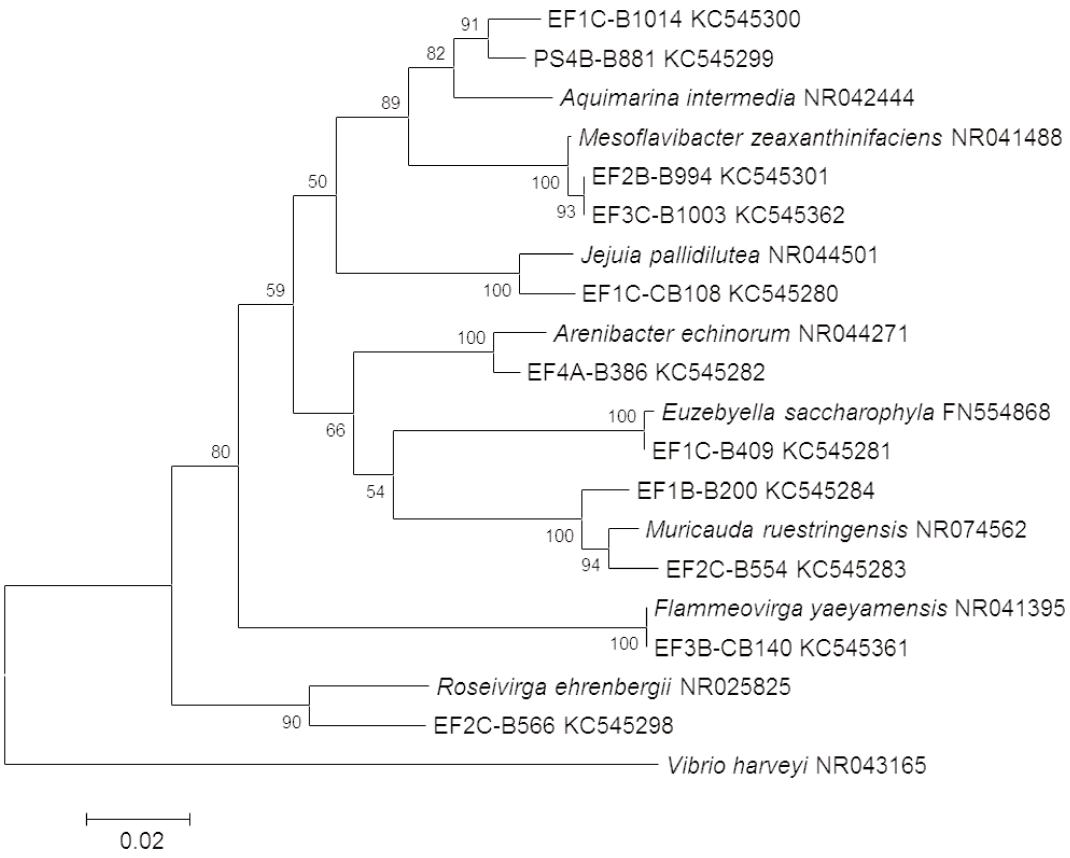


Figure 3.6 Phylogenetic tree of 20 *Plexauridae Flavobacteria* and *Cytophaga* isolates and related type strains based on 16S rRNA gene sequences. The evolutionary history was inferred using the Neighbor Joining method. Bar length = 2 substitutions/ 100 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch nodes (values <50% were excluded). The phylogenetic tree was constructed based on 336 positions. NR043165 was used as the outgroup to root the tree.

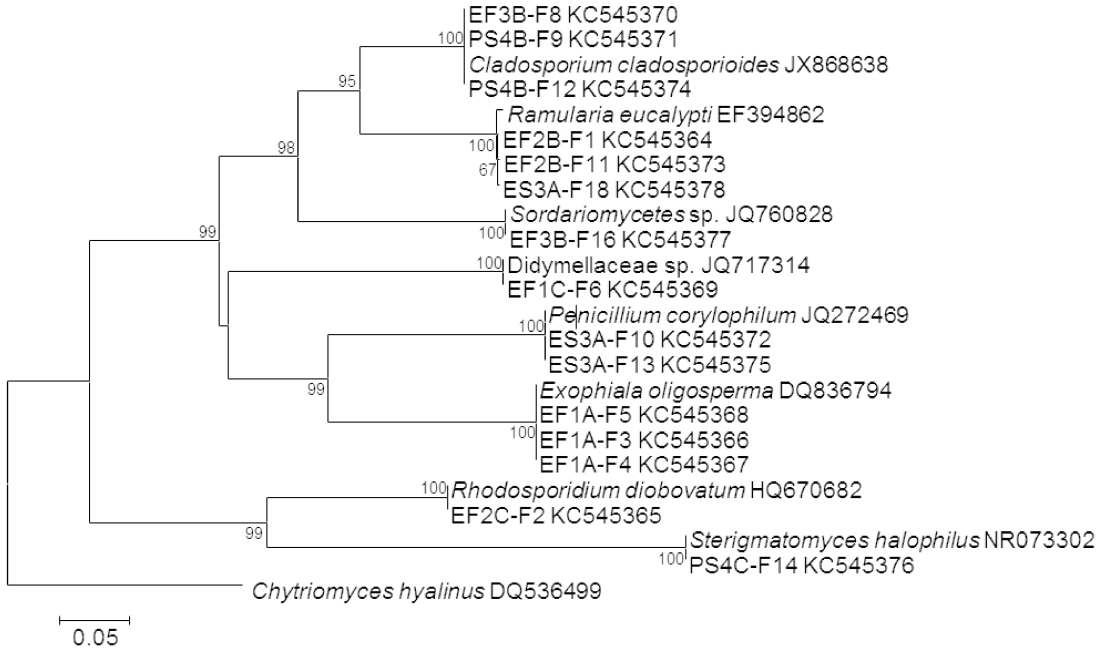


Figure 3.7 Phylogenetic tree of 24 *Plexauridae* fungi and related type strains based on rRNA/ITS gene sequences. The evolutionary history was inferred using the Neighbor Joining method. Bar length represents 5 substitutions/100 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch nodes (values <50% were excluded). The phylogenetic tree was constructed based on 319 positions. DQ536499 was used as the outgroup to root the tree.

Of the 148 unique microbes isolated from the 12 *Plexauridae* octocoral samples, 31 were novel bacteria (<97% 16S rDNA similarity to cultured strains in GenBank) that had not previously been formally characterized (Table 3.6, pp. 134-138, % ID blue and green boxes). Of these 31 bacteria, four (GenBank Acc. No.: JX488684, JX488685, KC545235, and KC545249) were novel species that had not previously been cultured (listed as "uncultured" or "clone" in GenBank), and four were novel genera (GenBank Acc. No.: KC545236, KC545284, KC545298, and KC545311) (<95% 16S rDNA identity to cultured strains in GenBank) that were never cultured. All fungi were previously cultured, although two of the 11 fungi (*Didymellaceae* fungi EF1C-F6 and *Sordariomycetes* fungi EF3B-F16) have likely not been formally characterized according to their ITS1-ITS4 sequence, but further sequence analysis (full-length sequencing of the 18S and 28S rDNA) should be carried out to confirm this statement.

3.3.2 Composition of Cultured Microbial Communities from Plexauridae Octocorals

From *E. fusca* (n=9), 120 unique microbes were cultured from nine samples. The number of unique microbes isolated from *Eunicea* sp., *Plexaura* sp. 1, and *Plexaura* sp. 2 (all n=1) were 14, 19, and 6, respectively. All *Plexauridae* were dominated by bacteria in the class *Gammaproteobacteria* (Figure 3.8 – 3.11). It was rare to find the same cultured microbe (16S rDNA sequence ≥99% identity) in more than one sample of *Plexauridae*, except for *Vibrio* and *Pseudoalteromonas* spp., which were ubiquitous members of all cultured libraries.

The only *Plexauridae* species with replicates (n=9) was *E. fusca*, so conclusions about the phylogenetic composition of cultured microbes can only be discussed for this octocoral. If just examining the compiled *E. fusca* cultured library (Figure 3.8), the composition by class was *Gammaproteobacteria* (42.5%), *Alphaproteobacteria* (20.0%), *Bacilli* (16.7%), *Actinobacteria* (7.5%), *Flavobacteriia* (6.7%), *Dothideomycetes* (2.5%), *Cytophaga* (1.7%), and *Eurotiomycetes*, *Microbotryomycetes*, and *Sordariomycetes* (0.8%). *Vibrio* and *Pseudoalteromonas* were the most abundant genera within the *Gammaproteobacteria*, and *Ruegeria*, *Bacillus*, and *Micrococcus* spp. were the most abundant genera in *Alphaproteobacteria*, *Bacilli*, and *Actinobacteria*, respectively.

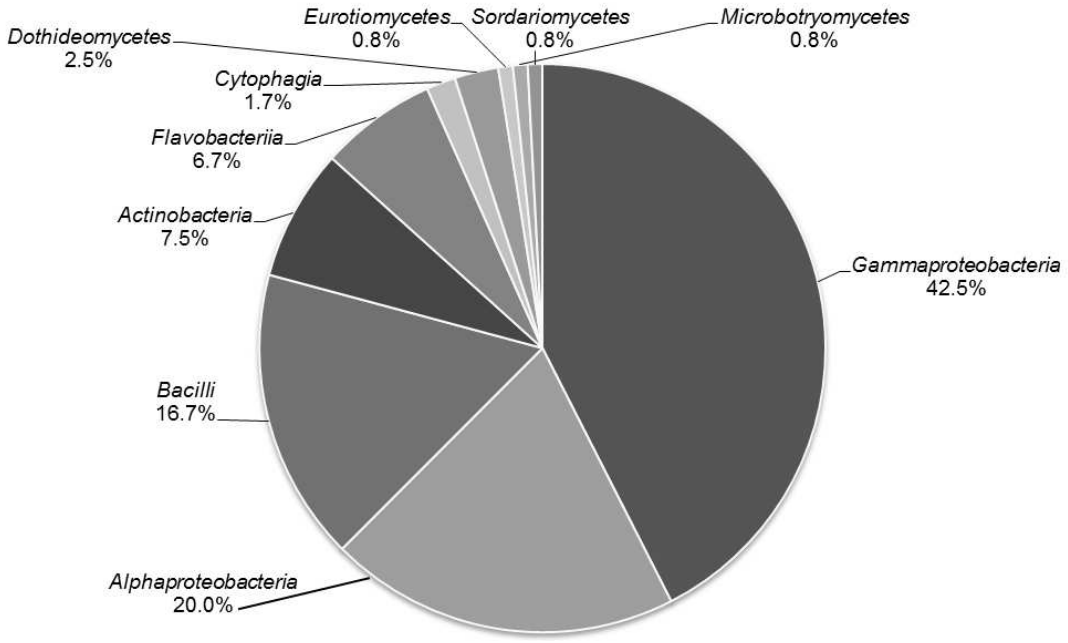


Figure 3.8 Culturable microbial diversity at the class-level of *Eunicea fusca* (n=9).

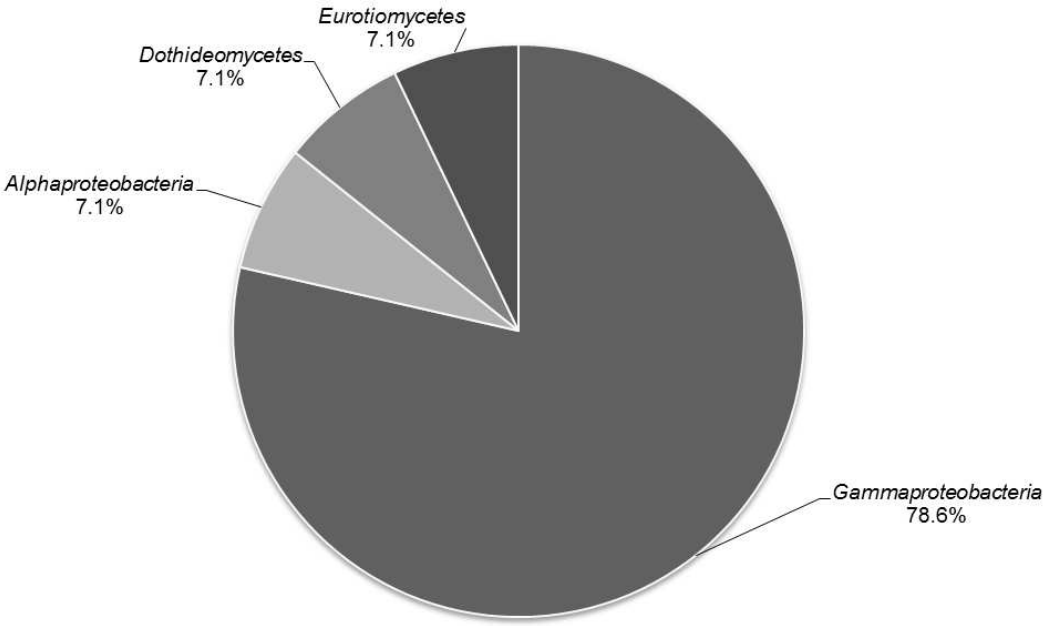


Figure 3.9 Culturable microbial diversity at the class-level of *Eunicea* sp. (n=1).

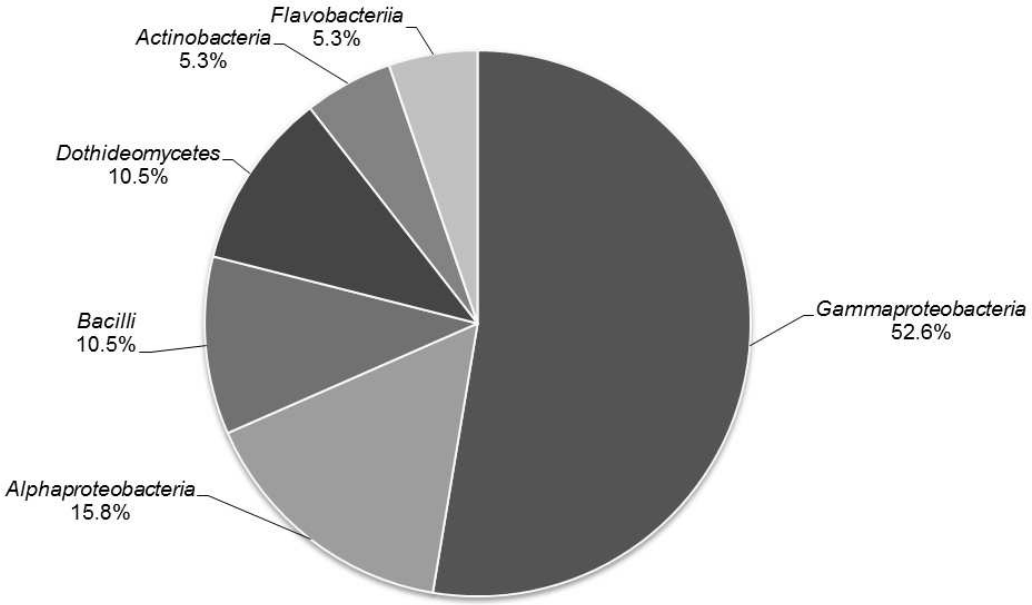


Figure 3.10 Culturable microbial diversity at the class-level of *Plexaura* sp. 1 (n=1).

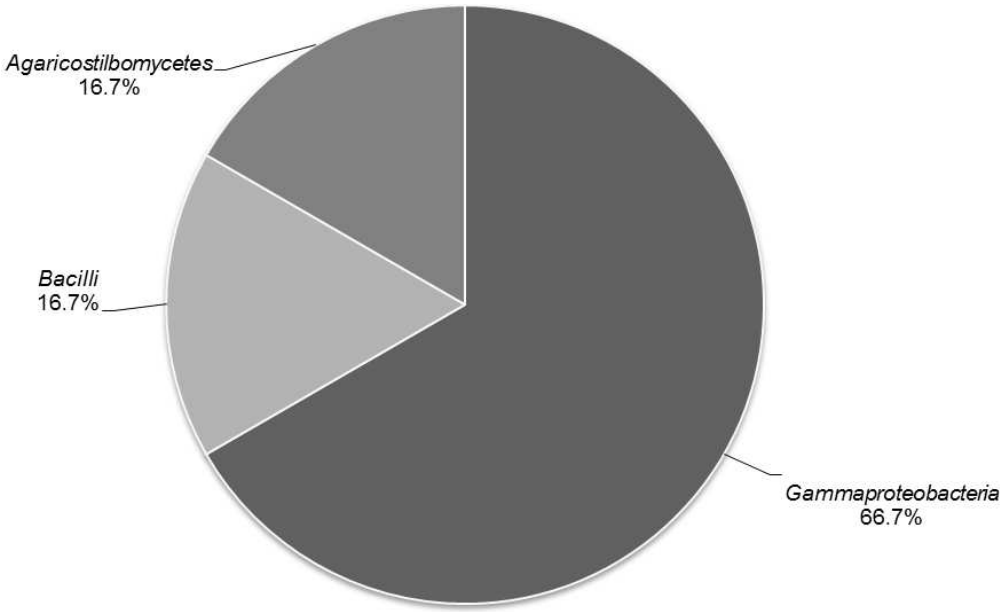


Figure 3.11 Culturable microbial diversity at the class-level of *Plexaura* sp. 2 (n=1).

3.3.3 Microbial Isolation Conditions

Correlations were observed between the percent of unique microbes isolated and methodological factors, such as particle size, initial dilution, media, and sampling site (Figure 3.12). Overall, the greatest number of unique microbes was isolated from sample EF-BS3-B; almost 10% more microbes were isolated from this *E. fusca* as compared to all other *Plexauridae*. Particle size also influenced the percent of microbes isolated; more than half (50.3%) of all microbes were isolated from the particle filtrate (<51 μm). Initial dilutions of >1-10^{-1} (34.6%) and >10^{-2}-10^{-3} (32.7%) yielded the highest percentage of unique microbes. The initial isolation media MA yielded almost half (46.4%) of the microbes, more than twice as many microbes as any other media. Most microbes (31.2%) were isolated from site BS3, 5-10% more microbes than any other site (Figure 3.12). If just considering fungi, more than half (53.3%) were isolated from undiluted particles, and 100% were isolated on YM (data not shown). *Plexauridae* sample, particle size, and site did not seem to influence the percentage of unique fungi isolated.

With regards to the correlations between the percent of novel microbes isolated and isolation factors, there were also some notable correlations (Figure 3.13). EF-FL2-C yielded the most novel microbes (22.6%). The particle filtrate (<51 μm) and <104 - ≥51 μm together made up 90.3% of the novel isolates. An initial dilution of >10^{-2}-10^{-3} yielded 41.9% of the novel bacteria. MA media yielded the most (61.3%) novel isolates, >40% more than any other media, and site FL2 gave the most (32.3%) novel bacteria (Figure 3.13).

There were also correlations between microbial genera and the isolation factors (Table 3.8). For example, all *Exophiala* spp. (n=3) were isolated from a <104 - ≥51 μm particle size, no dilution, on YM media, from site FL1. All *Enterovibrio* spp. (n=3) were isolated from the particle filtrate (<51 μm), at a dilution of 10^{-3}, from the BS sites.

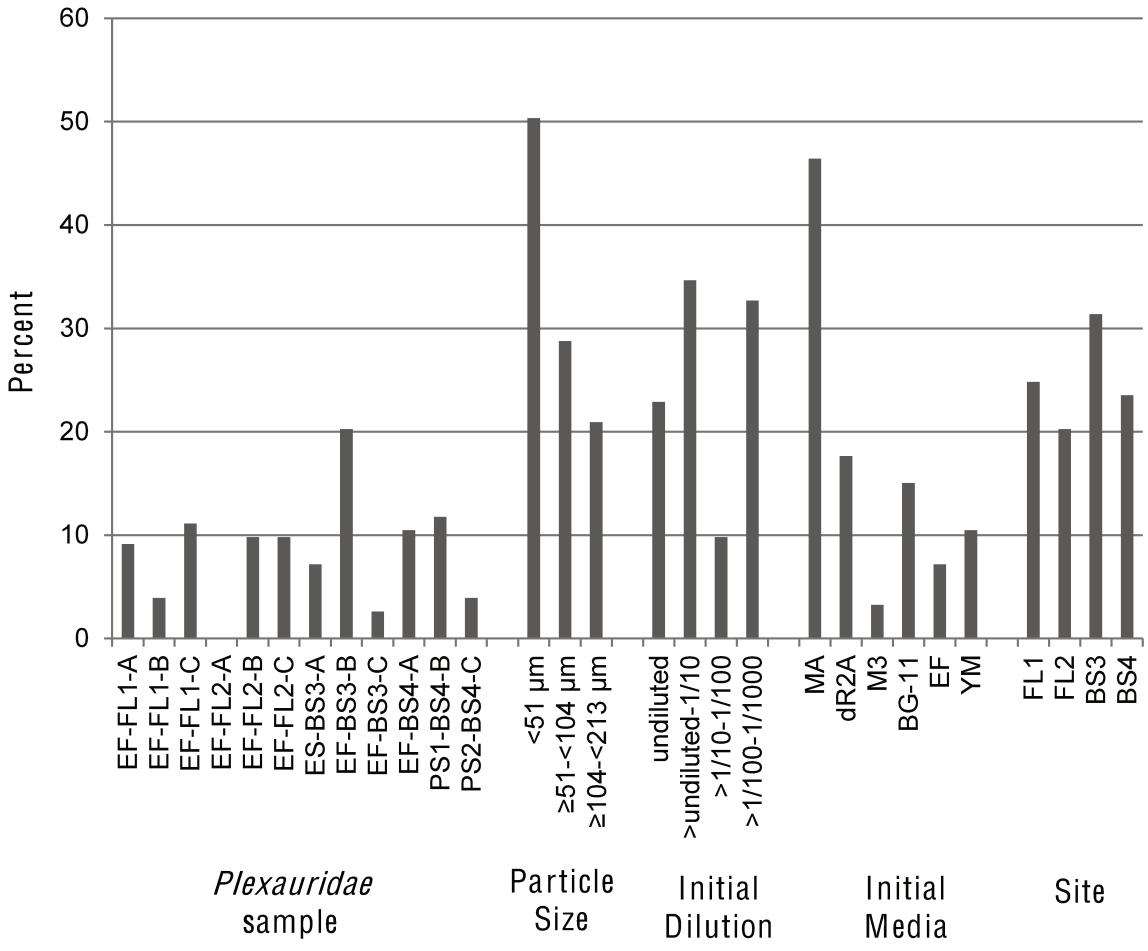


Figure 3.12 Comparison of initial isolation conditions for unique microbial isolates.
Abbreviations: EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; FL = Florida; BS = The Bahamas; A, B, C = Octocoral replicates at each individual site; MA = Marine Agar; dR2A = diluted R2A agar; EF = *Eunicea fusca*-specific agar; YM = modified yeast-malt agar; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4

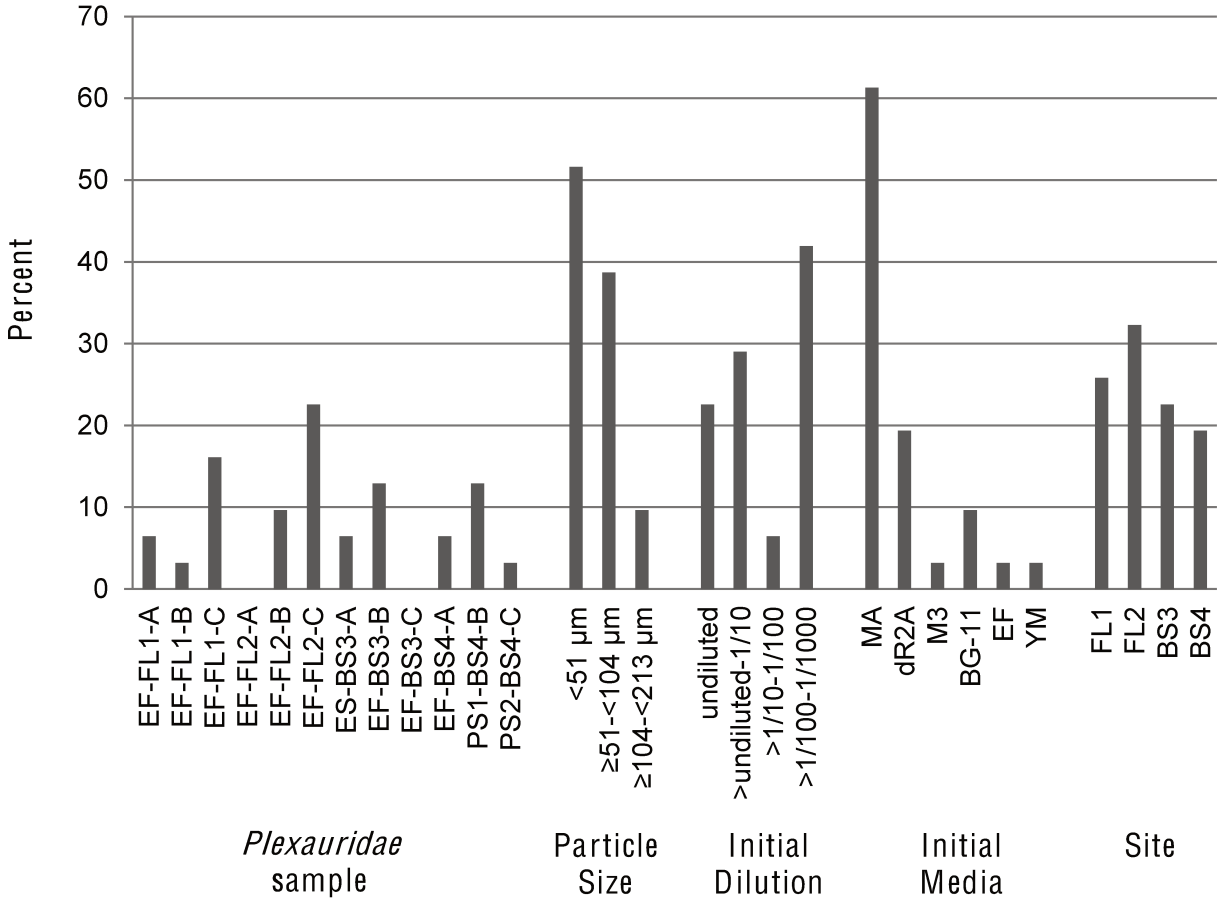


Figure 3.13 Comparison of initial isolation conditions for novel microbial isolates.
Abbreviations: EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; FL = Florida; BS = The Bahamas; A, B, C = Octocoral replicates at each individual site; MA = Marine Agar; dR2A = diluted R2A agar; EF = *Eunicea fusca*-specific agar; YM = modified yeast-malt agar; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4

Table 3.8 Correlations of microbial species to isolation conditions.

Microbial species (n=X)	Particle Size (% isolated under indicated conditions)	Dilution (% isolated under indicated conditions)	Media (% isolated under indicated conditions)	Site (% isolated under indicated conditions)
Bacteria				
Alcanivorax spp. (3)	--	--	MA (100%)	--
Alteromonas spp. (4)	--	--	--	BS (100%)
Amphritea spp. (2)	<51 μm (100%)	10^{-3} (100%)	--	--
Aquimarina spp. (2)	≥51-<104 μm (100%)	--	dR2A (100%)	--
Bacillus spp. (16)	<104 μm (87.5%)	--	--	--
Endozoicomonas spp. (3)	--	--	MA (100%)	--
Enterovibrio spp. (3)	<51 μm (100%)	10^{-3} (100%)	--	BS (100%)
Halomonas spp. (2)	--	--	dR2A (100%)	--
Labrenzia spp. (4)	<104 μm (100%)	--	--	--
Mesoflavibacter spp. (2)	--	--	MA (100%)	--
Micrococcus spp. (4)	<104 μm (100%)	--	--	--
Muricauda spp. (2)	≥51-<104 μm (100%)	--	MA (100%)	FL (100%)
Oceanobacillus spp. (3)	<51 μm (100%)	10^{-3} (100%)	MA (100%)	--
Photobacterium spp. (3)	<51 μm (100%)	--	--	--
Pseudoalteromonas spp. (11)	--	--	MA (63.6%)	BS (72.7%)
Ruegeria spp. (12)	<104 μm (83.3%)	--	--	FL (83.3%)
Shewanella spp. (3)	<104 μm (100%)	--	MA (100%)	BS3 (100%)
Staphylococcus spp. (2)	<104 μm (100%)	$1\text{-}10^{-1}$ (100%)	MA (100%)	FL (100%)
Vibrio spp. (27)	--	$1\text{-}10^{-1}$ (74.0%)	*Not* MA (70.1%)	--
Fungi				
Cladosporium spp. (3)	<51 μm (100%)	10^{-3} (100%)	YM (100%)	BS (100%)
Exophiala spp. (3)	≥51-<104 μm (100%)	1 (100%)	YM (100%)	FL1 (100%)
Penicillium spp. (2)	≥104 μm (100%)	1 (100%)	YM (100%)	BS3 (100%)

Abbreviations: MA = Marine Agar; dR2A = diluted R2A agar; EF = *Eunicea fusca*-specific agar; YM = modified yeast-malt agar; BS = The Bahamas; FL = Florida; BS3 = BS site 3; FL1 = FL site 1

3.3.4 Comparison of Cultured Microbial Community to Culture-Independent Library

In this study, it was found that 74 (54.0%) of the cultured bacterial sequences were also found in the culture-independent library (Chapter 2) constructed in a parallel study (Table 3.9). These 74 sequences had ≥97% sequence similarity between the two libraries. Some of the sequences were retrieved from the same octocoral samples (*e.g. Bacillus* sp. EF1B-B343, *Pelagibius* sp. EF2B-B188, *Endozoicomonas* sp. PS125, *Vibrio* sp. PS4C-CB169, and *Streptomyces* sp. EF3B-B867) or *Plexauridae* from the same site (*e.g. Staphylococcus* sp. EF2B-B65 and *Kiloniellaceae* sp. EF3B-B119). More than half (59.5%) of the similar cultured bacteria, however, were most similar to sequences found in the surrounding seawater column from the culture-independent library. No cultured fungi had similar relatives in the culture-independent library.

Although half of the bacteria had closely-related counterparts in the culture-independent library, in reality, the percent of the total microbial community isolated was quite low. If just comparing the *E. fusca* generic (95% 16S rDNA sequence identity) overlap, only 21 of the 206 "total" culture-independent *E. fusca* genera (10.1%) were isolated. At the strain-level (≥99% rDNA sequence similarity), only 7 (0.56%) cultured sequences of the 1,261 unique, culture-independent *E. fusca* sequences (compilation of all dereplicated, culture-independent *E. fusca* sequences) were cultured.

Table 3.9 Comparison of cultured *Plexauridae* microbes to the culture-independent (combined 454-pyrosequencing and denaturing gradient gel electrophoresis) library. Only sequences with ≥97% 16S rDNA sequence similarity to culture-independent library are reported.

GenBank Acc. No.	GenBank ID	ID of closest match in culture-independent library	% ID	Notes
KC545234	*Pseudoalteromonas* sp. EF1B-B175	FLW1_GBHUE1304JDFV3	98	Both from FL1
KC545235	*Aestuariibacter* sp. ES3A-B772	FLW1_GBHUE1304I2NZK	97	
KC545237	*Alteromonas* sp. EF3B-CB141	FLW2_GBHUE1304JC4JQ	98	
KC545239	*Alteromonas* sp. PS4B-B104	BHW2U7302ERO6V	99	Both from BS4
KC545245	*Alcanivorax* sp. PS4B-B178	FLW2_GBHUE1304ILFAR	97	
KC545250	*Staphylococcus* sp. EF1A-B1042	EF2A_U7302D4675	97	Both from FL EF
KC545251	*Halomonas* sp. ES3A-B328	BHW2U7302C65AO	98	Both from BS
KC545253	*Ruegeria* sp. EF3B-B312	FLW1_GBHUE1304JEKJR	98	
KC545254	*Ruegeria* sp. EF2B-CB79	FLW1_GBHUE1304JEKJR	98	Both from FL
KC545255	*Staphylococcus* sp. EF2B-B65	EF2A_U7302D4675	99	Both from FL2 EF
KC545258	*Ruegeria* sp. EF2C-B768	FLW1_GBHUE1304JEKJR	98	Both from FL
KC545260	*Ruegeria* sp. EF2C-B216	FLW1_GBHUE1304JAWUM	99	Both from FL
KC545261	*Ruegeria* sp. EF2C-B1020	FLW1_GBHUE1304JEKJR	97	Both from FL
KC545262	*Ruegeria* sp. EF2C-B912	FLW1_GBHUE1304JAWUM	99	Both from FL
KC545264	*Ruegeria* sp. EF2C-B774	FLW1_GBHUE1304JAWUM	99	Both from FL
KC545265	*Pseudoruegeria* sp. EF2C-B818	FLW1_GBHUE1304JAWUM	98	Both from FL
KC545266	*Ruegeria* sp. EF1C-CB31	FLW1_GBHUE1304JEKJR	98	Both from FL1
KC545267	*Ruegeria* sp. EF2B-B203	FLW1_GBHUE1304JEKJR	97	Both from FL
KC545269	*Ruegeria* sp. EF1C-B905	FLW1_GBHUE1304JAWUM	98	Both from FL1
KC545270	*Bacillus* sp. EF1B-B343	EF1B_U7302EJZSU	97	Both from EF-FL1-B
KC545285	*Labrenzia* sp. EF1C-B300	EF3C_U7302D96K9	100	
KC545286	*Ornithinimicrobium* sp. EF2C-B618	DGGE_Jul_21_EF_3B_2	98	
KC545287	*Pseudovibrio* sp. PS4B-B512	FLW1_GBHUE1304IM71O	97	
KC545288	*Labrenzia* sp. EF3B-B762	EF1C_U7302DLSJ8	99	
KC545289	*Labrenzia* sp. ES3A-B1008	EF3C_U7302EG91A	98	Both from BS3
KC545290	*Labrenzia* sp. PS4B-B940	EF3C_U7302ECTTW	99	Both from BS
KC545296	*Shimia* sp. EF3B-CB115	FLW1_GBHUE1304JIE9B	97	
KC545297	*Pseudoalteromonas* sp. PS4C-CB146	BHW2U7302DEO49	99	Both from BS4
KC545302	*Ruegeria* sp. EF1C-B951	FLW2_GBHUE1304IEO39	99	Both from FL
KC545306	Rhodospirillaceae bacterium PS4B-B496	EF2B_U7302C7KK4	98	
KC545308	*Pelagibius* sp. EF2B-B188	EF2B_U7302C7KK4	97	Both from EF-FL2-B
JX488684	*Endozoicomonas* sp. EF212 (EF2C)	EF1C_U7302EJL67	99	Both from FL EF
JX488685	*Endozoicomonas* sp. PS125 (PS4B)	EF4B_U7302DV50P	99	Both from PS1-BS4-B
KC545311	Kiloniellaceae bacterium EF3B-B119	EF3C_U7302C9OUO	98	Both from BS3 EF
KC545312	*Vibrio* sp. EF4A-B1045	FLW1_GBHUE1304JGKS6	98	
KC545314	*Shewanella* sp. EF3B-B556	FLW2_GBHUE1304JL92K	99	
KC545315	*Shewanella* sp. EF3B-B536	FLW2_GBHUE1304JL92K	98	
KC545318	*Enterovibrio* sp. EF4A-B638	FLW1_GBHUE1304JFFNA	97	
KC545321	*Vibrio* sp. EF3B-CB164	FLW1_GBHUE1304IN1Q9	97	

KC545322	*Vibrio* sp. EF1A-B366	EF2C_U7302DE7IJ	98	Both from FL EF
KC545323	*Vibrio* sp. EF1A-B655	EF2C_U7302DE7IJ	98	Both from FL EF
KC545324	*Vibrio* sp. EF1A-B809	EF2C_U7302DE7IJ	98	Both from FL EF
KC545325	*Vibrio* sp. ES3A-B421	EF2C_U7302DE7IJ	98	
KC545326	*Vibrio* sp. EF3B-CB181	EF2C_U7302DE7IJ	98	
KC545327	*Vibrio* sp. EF3B-B689	EF2C_U7302DE7IJ	99	
KC545328	*Vibrio* sp. EF4A-CB189	EF2C_U7302DE7IJ	99	
KC545329	*Vibrio* sp. EF3B-CB191	EF2C_U7302DE7IJ	98	
KC545330	*Vibrio* sp. EF3B-B84	FLW1_GBHUE1304JGKS6	98	
KC545331	*Vibrio* sp. EF1C-CB45	FLW1_GBHUE1304JGKS6	98	Both from FL1
KC545332	*Vibrio* sp. EF1C-CB167	FLW1_GBHUE1304JGKS6	98	Both from FL1
KC545333	*Vibrio* sp. EF1C-B793	FLW1_GBHUE1304JGKS6	97	Both from FL1
KC545334	*Vibrio* sp. EF3B-CB182	FLW1_GBHUE1304JGKS7	97	
KC545335	*Vibrio* sp. EF3B-CB161	FLW1_GBHUE1304JGKS6	97	
KC545336	*Vibrio* sp. EF1C-B615	EF4C_U7302ERN4L	98	
KC545337	*Vibrio* sp. EF2B-B978	FLW1_GBHUE1304JGKS6	99	Both from FL
KC545338	*Vibrio* sp. EF3B-B166	EF2C_U7302DE7IJ	99	
KC545339	*Vibrio* sp. EF4A-CB199	FLW1_GBHUE1304JGKS6	97	
KC545340	*Vibrio* sp. ES3A-B773	FLW1_GBHUE1304JGKS6	97	
KC545341	*Photobacterium* sp. ES3A-B76	EF3B_U7302D4NLZ	98	Both from BS3
KC545343	*Vibrio* sp. ES3A-CB178	EF2C_U7302DE7IJ	97	
KC545344	*Vibrio* sp. EF4A-B696	EF4C_U7302ERN4L	99	Both from BS4
KC545345	*Vibrio* sp. PS4C-CB169	EF4C_U7302ERN4L	99	Both from PS2-BS4-C
KC545346	*Pseudoalteromonas* sp. EF1B-CB130	FLW1_GBHUE1304JDFV3	99	Both from FL1
KC545347	*Pseudoalteromonas* sp. PS4B-B484	BHW2U7302DEO49	99	Both from BS4
KC545348	*Pseudoalteromonas* sp. EF1A-B164	BHW2U7302DEO49	99	
KC545349	*Pseudoalteromonas* sp. EF4A-B537	FLW2_GBHUE1304JQACK	97	
KC545350	*Pseudoalteromonas* sp. EF3B-B799	FLW1_GBHUE1304JDFV3	97	
KC545351	*Pseudoalteromonas* sp. EF3B-B588	FLW1_GBHUE1304JDFV3	97	
KC545352	*Pseudoalteromonas* sp. EF3B-B256	FLW1_GBHUE1304JDFV3	97	
KC545353	*Pseudoalteromonas* sp. PS4B-B1021	FLW1_GBHUE1304IWPK3	99	
KC545359	*Streptomyces* sp. EF3B-B867	Jul_21_EF_3B_2	98	Both from EF-BS3-B
KC545363	*Pseudoalteromonas* sp. EF3B-F15	BHW2U7302DEO49	99	Both from BS

Abbreviations: EF = *Eunicea fusca*; ES = *Eunicea* sp.; PS1, PS2 = *Plexaura* spp. 1 and 2; FL = Florida; BS = The Bahamas; A, B, C = Octocoral replicates at each individual site; FL1 = FL site 1; FL2 = FL site 2; BS3 = BS site 3; BS4 = BS site 4

3.3.5 Investigating Plexauridae-Associated Bacteria for Novel Antimicrobials and Fuscol

3.3.5.1 Antibiosis Study with *Endozoicomonas* spp.

Endozoicomonas spp. constituted a large portion of the culture-independent library (Chapter 2), and two novel isolates (EF212^T, GenBank Acc. No. JX488684; PS125^T, GenBank Acc. No. JX488685) of this genus (that had >99% 16S rDNA sequence identity to members of the culture-independent library) were cultured in this study. Therefore, it was of interest to further explore the secondary metabolism of these ubiquitous and abundant members of the *Plexauridae* microbiomes.

The first study carried out explored their biological interactions with all other *Plexauridae* cultured bacteria. In this cross-streak antibiosis study, it was found that EF212^T and PS125^T did not display any inhibitory activity or phenotypic changes against all other *Plexauridae* isolates. However, six bacterial isolates exhibited reproducible bioactivity against the *Endozoicomonas* spp. (Table 3.10). *Euzebyella* sp. EF1C-B409 produced an interesting "rhizoid" structure, and *Enterovibrio* sp. EF4A-CB187 produced a red-green iridescent pigment in the confluence zone with both *Endozoicomonas* spp. The *Pseudoalteromonas* spp. EF4A-B537 and EF3B-B588 and *Streptomyces* sp. EF3B-B867 all inhibited the growth of the *Endozoicomonas* spp. in the confluence zone, and inhibition zones were measured in a subsequent overlay of the inhibitory strains on the *Endozoicomonas* spp. (Table 3.10).

Table 3.10 Cultured *Plexauridae* bacteria that demonstrated bioactivity against *Endozoicomonas* EF212^T (JX488684) and *Endozoicomonas* PS125^T (JX488685).

GenBank Acc. No.	GenBank ID	Description of activity against *Endozoicomonas* spp.	Zone of Inhibition (mm)
KC545281	*Euzebyella* sp. EF1C-B409	Produced "rhizoid" structure towards *Endozoicomonas* spp.	N/A
KC545319	*Enterovibrio* sp. EF4A-CB187	Produced "red-green" iridescence towards *Endozoicomonas* spp.	N/A
KC545294	*Bacillus* sp. EF4A-B1046	Growth inhibition of *Endozoicomonas* spp.	EF212^T: 2 mm; PS125^T: 1 mm
KC545349	*Pseudoalteromonas* sp. EF4A-B537	Growth inhibition of *Endozoicomonas* spp.	EF212^T: 5 mm; PS125^T: 2 mm
KC545351	*Pseudoalteromonas* sp. EF3B-B588	Growth inhibition of *Endozoicomonas* spp.	EF212^T: 8 mm; PS125^T: 2 mm
KC545359	*Streptomyces* sp. EF3B-B867	Growth inhibition of *Endozoicomonas* spp.	EF212^T and PS125^T reduced growth, hazy inhibition zone

Abbreviations: N/A = not applicable; Acc. No. = accession number; ID = identity

3.3.5.2 Fermentation of *Euzebyella* sp. EF1C-B409, Compound Isolation, Structural Elucidation, and Bioactivity Screening

Euzebyella sp. EF1C-B409 (KC545281) demonstrated interesting phenotypic changes in close proximity to *Endozoicomonas* spp. EF212^T and PS125^T and was therefore further explored for the production of antimicrobial MNPs through fermentation, chemical profiling, and antimicrobial bioassays.

The chemistry of *Euzebyella* sp. EF1C-B409 was further investigated through fermentations followed by bioassay- and chemical-guided fractionation of a novel, antimicrobial compound. HPLC purification led to the isolation of a novel compound identified as 2-isononyl-5-isobutylresorcinol ($C_{19}H_{32}O_2$; MW=292.45618). The structural elucidation was accomplished by the interpretation of the ^{1}H, COSY, HSQC, and HMBC NMR experiments (Figure 3.14, Table 3.11, and APPENDIX A: Supplementary Figure 3.1, p. 274), LC-HRMS (Figure 3.15), and HRMS-MS (Figure 3.16).

The ^{1}H NMR spectrum (APPENDIX A: Supp. Figure 3.1, p. 274) of 2-isononyl-5-isobutylresorcinol indicated that there were two identical aromatic protons (H_4/H_6; δ6.11, 2H, s), one methylene ($H_{1'}$; δ2.54, t, J = 7.7 Hz, 2H), one methylene ($H_{1''}$; δ2.26, d, J = 7.2 Hz, 2H), unresolved methylenes (δ1.17-1.49; 10 H), unresolved methines (δ1.52-1.80; 2 H), and two pairs of equivalent methyls (δ0.88, 6H; δ0.87, 6H). Two-dimensional NMR experiments further confirmed the structure. ^{1}H-^{13}C HMBC long-range couplings (Figure 3.14 and Table 3.11) that were used to establish the presence of the 2,5-dialkylresorcinol moiety were as follows: from two equivalent H_4/H_6 aromatic protons (δ6.11) to C_1/C_3 (δ156.8), C_2 (δ114.5), C_5 (δ140.9) and $C_{1''}$ methylene (δ46.4); from $H_{1'}$ benzylic protons (δ2.54, t) to C_1/C_3 (δ156.8), C_2 (δ114.5), and $C_{2'}$ (δ30.1); and from $H_{1''}$ benzylic protons (δ2.26, d) to C_4/C_6 (δ108.3) and C_5 (δ140.9). Since two equivalent methyl protons at δ0.87 ($H_{8'}/H_{9'}$) were coupled with the $C_{6'}$ methylene (δ40.1) and $C_{7'}$ methine (δ29.0) carbons, and the two other equivalent methyl protons at δ0.88 ($H_{3''}/H_{4''}$) were coupled with the $C_{1''}$ methylene (δ46.4) and $C_{2''}$ methine (δ31.0) carbons, the alkyl side chains

were deduced to be of the iso-alkyl type. HMBC long-range coupling from the aromatic protons H_4/H_6 (δ6.11) to the $C_{1''}$ methylene carbon (δ46.4), as well as a COSY coupling from the H_4/H_6 protons to the $H_{1''}$ (δ2.26) protons, proved that the alkyl side chain attached at C_5 on the resorcinol ring was an isobutyl residue, and consequently, the alkyl side chain attached at C_2 on the resorcinol ring was deduced to be an isononyl residue.

This structure was further confirmed through LC-HRMS (Figure 3.15) using negative chemical ionization with a $[M - H]^-$ m/z 291.23303 (calculated for $C_{19}H_{31}O_2^-$, 291.23241, Δ 2.1 ppm). Finally, the HRMS-MS experiments (Figure 3.16) using negative chemical ionization confirmed the length of the two alkyl side chain fragment ions with the m/z 248.17818 fragment interpreted as cleavage of the isopropyl group on the isobutyl side chain with further loss of the isopropyl group on the isononyl side chain (m/z 205.12340), and the m/z 165.09210 fragment interpreted as cleavage of the isononyl side chain.

This compound showed good Gram-positive activity against MRSA, VRE, and *S. warneri* as compared to the positive control antibiotics, vancomycin and rifampicin (Table 3.12 and Figure 3.17).

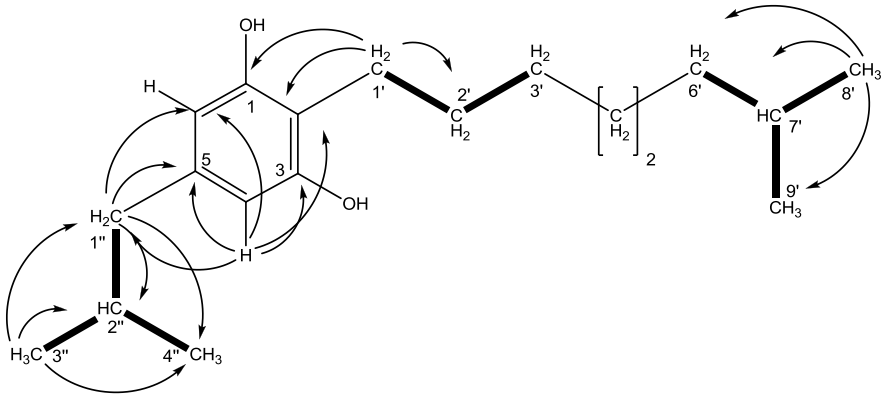


Figure 3.14 Key COSY (bold lines) and HMBC (arrows) correlations for the novel compound, 2-isononyl-5-isobutylresorcinol, isolated from *Euzebyella* sp. EF1C-B409.

Table 3.11 Nuclear magnetic resonance data for 2-isononyl-5-isobutylresorcinol.[a]

Position	**δC, Type**	**δH, mult. (*J* in Hz)**	COSY	HMBC
1, 3	156.8, C	--	--	
2	114.5, C	--	--	
4,6	108.3, CH	6.11 s	$H_{1''}$	C_1/C_3, C_2, C_4/C_6, C_5, $C_{1''}$
5	140.9, C	--	--	
1’	23.8, CH_2	2.54 t (7.7)	$H_{2'}$	C_1/C_3, C_2, $C_{2'}$
2’	30.1, CH_2	1.49 m	$H_{1'}$, $H_{3'}$	
3’	30.6, CH_2	1.34 m	$H_{2'}$, $H_{4'}$	
4’	30.7[b], CH_2	1.30 m	$H_{3'}$	
5’	28.4[b], CH_2	1.30 m	$H_{5'}$	
6’	40.1, CH_2	1.17 m	$H_{5'}$, $H_{7'}$	
7’	29.0, CH	1.52 m	$H_{6'}$, $H_{8'}/H_{9'}$	
8’/9’	22.8, CH_3	0.87 m	H-7’	$C_{6'}$, $C_{7'}$, $C_{8'}/C_{9'}$
1”	46.4, CH_2	2.26 d (7.2)	H_4/H_6, $H_{2''}$	C_4/C_6, C_5, $C_{2''}$, $C_{3''}/C_{4''}$
2”	31.0, CH	1.80 m	$H_{1''}$, $H_{3''}/H_{4''}$	$C_{3''}/C_{4''}$
3”/4’’	22.7, CH_3	0.88 m	$H_{2''}$	$C_{1''}$, $C_{2''}$, $C_{3''}/C_{4''}$

[a]Measured at 600 MHz (^{1}H) and 150 MHz (^{13}C) in CD_3OD.
[b]Interchangeable.
Abbreviations: mult. = multiplicity; Hz = hertz; COSY = correlation spectroscopy; HMBC = heteronuclear multiple bond correlation; s = singlet; m = multiplet; d = doublet; t = triplet

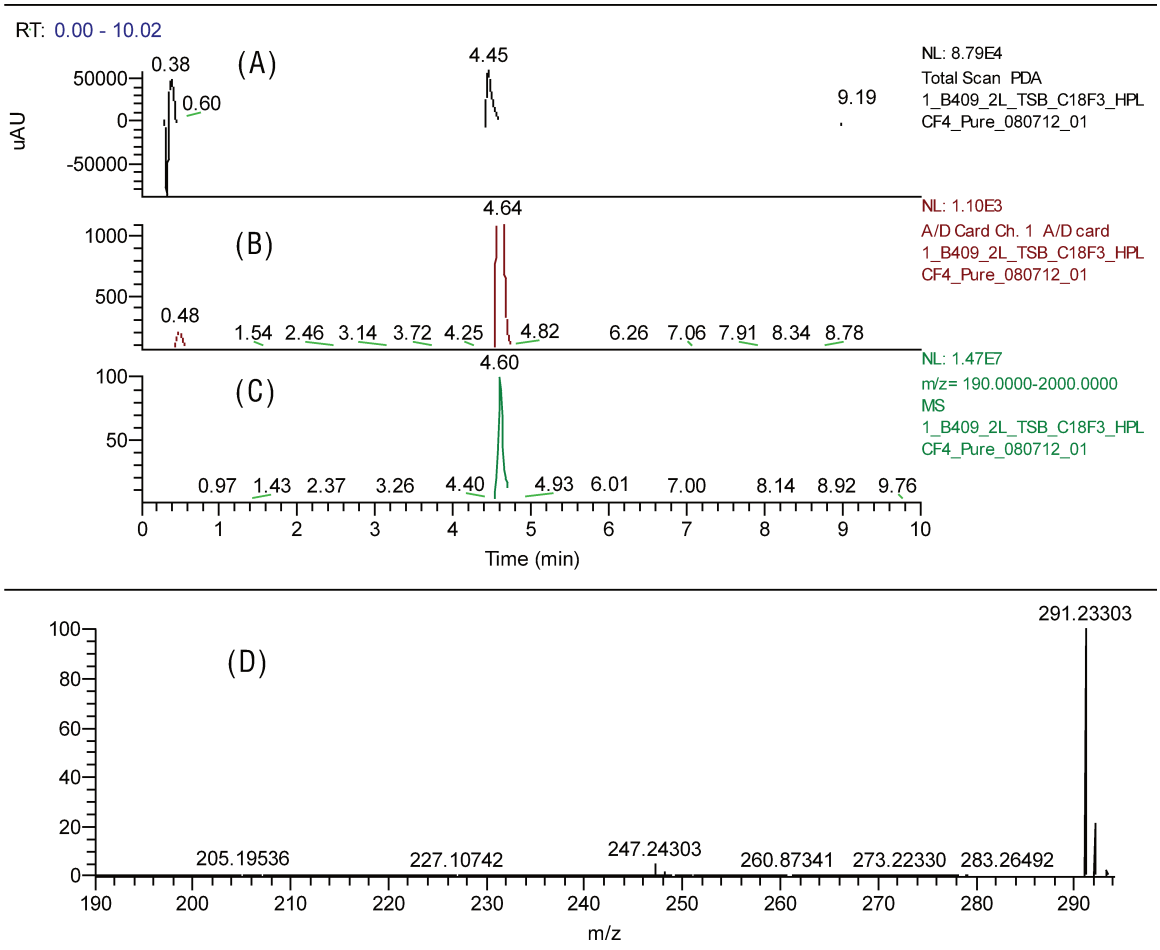


Figure 3.15 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) profile of pure 2-isononyl-5-isobutylresorcinol. (A) UV, (B) Evaporative-light scattering detector (ELSD), and (C) Mass spectrometry (MS) (negative ionization) retention time (RT) profile. (D) Mass spectrum (190.00-300.00 m/z) at a RT of 4.64. $[M - H]^-$ m/z 291.23303 (calculated for $C_{19}H_{31}O_2^-$, 291.23241, Δ 2.1 ppm)

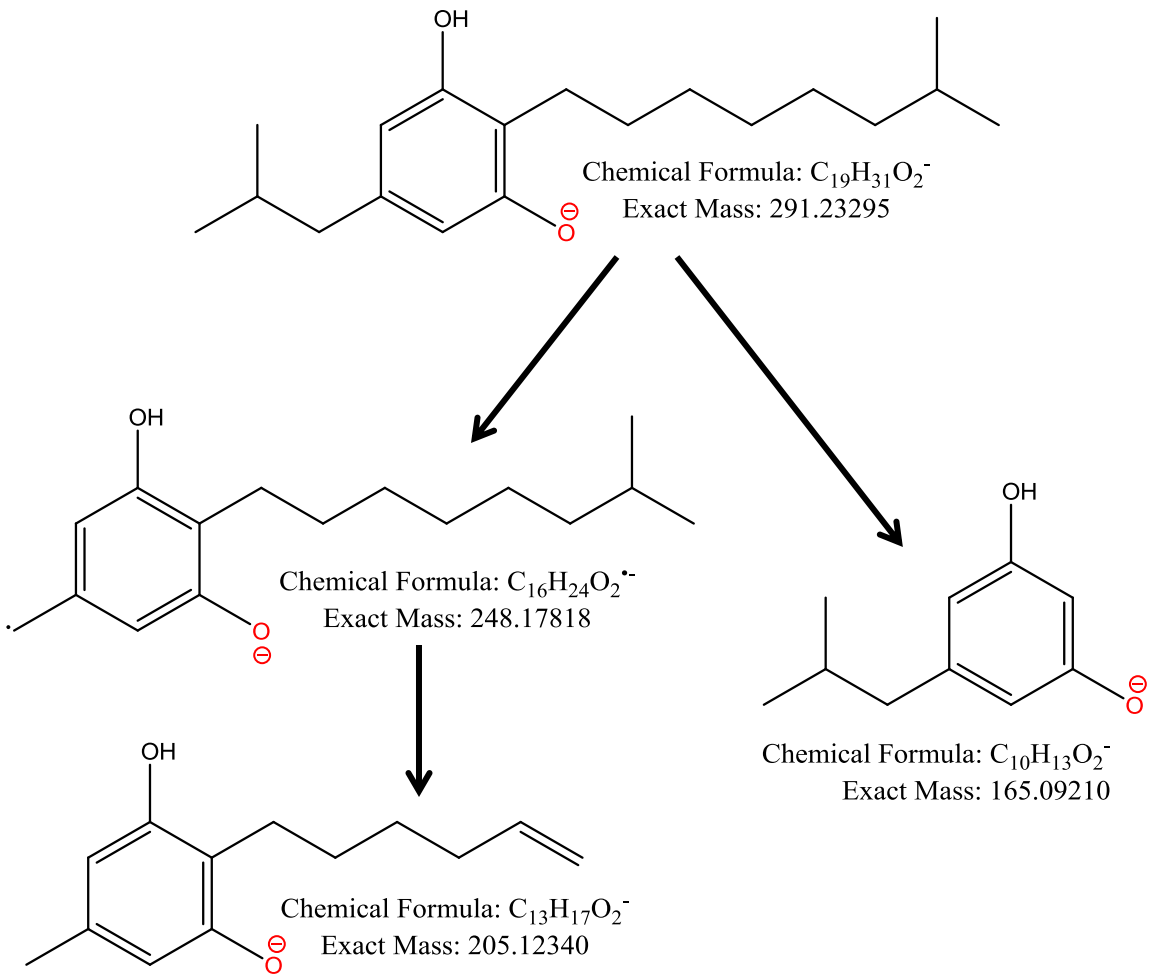


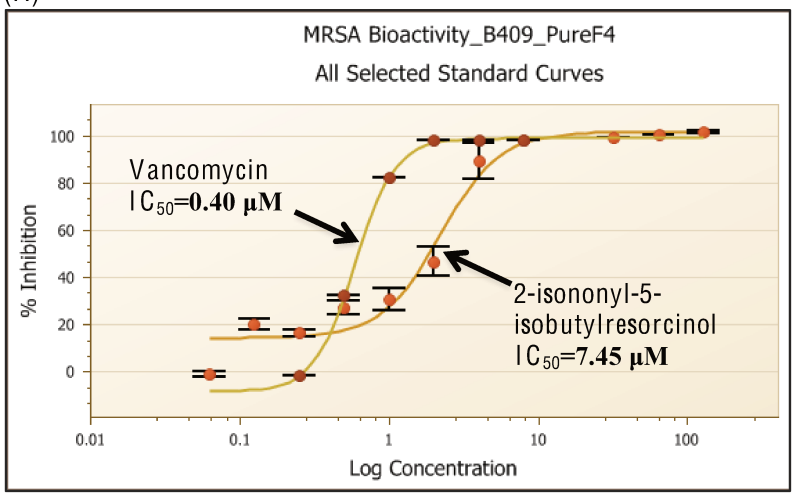
Figure 3.16 High resolution mass spectrometry-mass spectrometry (HRMS-MS) (negative ionization) fragmentation pattern of 2-isononyl-5-isobutylresorcinol. Functional groups are shown in red. (-) indicates a negative charge.

Table 3.12 IC_{50} and MIC values for the novel marine natural product isolated from *Euzebyella* sp. EF1C-B409 and control compounds.

	MRSA		VRE		*S. warneri*	
	IC_{50}	MIC	IC_{50}	MIC	IC_{50}	MIC
2-isononyl-5-isobutylresorcinol	7.5	13.7	7.4	13.7	20.8	27.4
vancomycin control	0.4	1.4			0.4	0.7
rifampicin control			0.9	2.4		

Abbreviations: IC_{50} = half maximum inhibitory concentration; MIC = minimum inhibitory concentration; MRSA = methicillin-resistant *Staphylococcus aureus*; VRE = vancomycin-resistant *Enterococci*; *S. warneri* = *Staphylococcus warneri*

(A)



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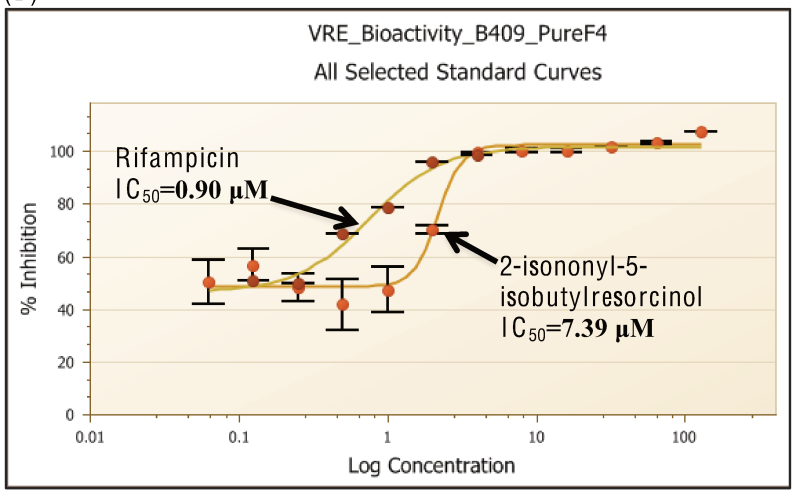


(C)

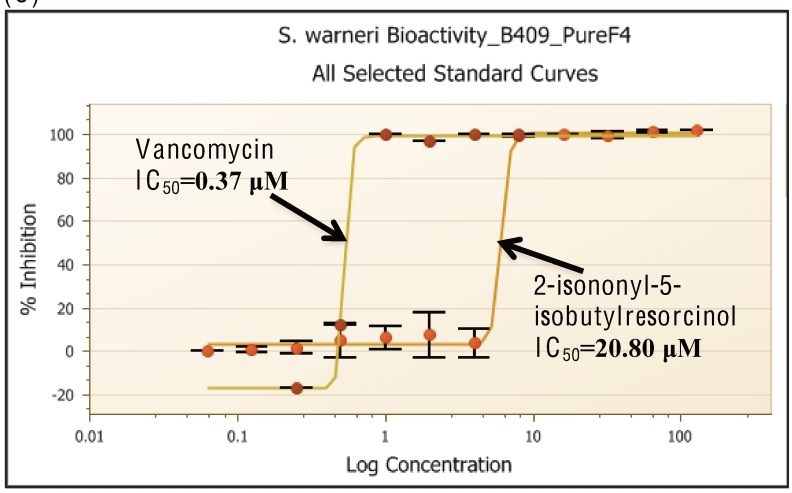


Figure 3.17 Inhibition curves for 2-isononyl-5-isobutylresorcinol and positive controls (vancomycin and rifampicin) against (A) methicillin-resistant *Staphylococcus aureus* (MRSA), (B) vancomycin-resistant *Enterococci* (VRE), and (C) *S. warneri*. Each point represents the mean of three independent experiments ± S.E.M. (standard error of the mean). The half maximal (50%) inhibitory concentrations (IC_{50}) were determined for the novel compound and controls.

3.3.5.3 Fermentation of *Endozoicomonas* spp. EF212^T and PS125^T and Chemical and Bioactivity Screening of Extracts

Fuscol was not detected by LC-HRMS in *Endozoicomonas* spp. crude EtAOc fermentation extracts, and no antimicrobial activity was detected in crude EtOAc or H_2O fermentation extracts at a concentration of 50 μg ml^{-1}. Moreover, no interesting ions (*i.e.* novel ions not found in Antibase) were detected by LC-HRMS, so these bacteria were not explored any further for the production of MNPs in this study.

3.3.5.4 Fermentation of *Labrenzia* sp. EF3B-B762, Compound Isolation, Structural Elucidation, and Bioactivity Screening

The chemistry of *Labrenzia* sp. EF3B-B762 was further investigated through fermentations followed by bioassay- and chemical-guided fractionation. HPLC purification led to the isolation of two novel fatty acid derivatives, (*E*)-10-oxooctadec-11-enoic acid ($C_{18}H_{32}O_3$; MW=296.44488) (Figure 3.18) and (*Z*)-10-oxooctadec-11-enoic acid ($C_{18}H_{32}O_3$; MW=296.44488) (Figure 3.19). Structural elucidation of these fatty acid derivatives was accomplished by the interpretation NMR (^{1}H, COSY, HSQC, and HMBC experiments) (Tables 3.13 and 3.14; APPENDIX A: Supp. Figure 3.2, p. 275; Supp. Figure 3.3, p. 276), LC-HRMS (Figures 3.20 and 3.21), and HRMS-MS (Figure 3.22).

The ^{1}H NMR spectrum (APPENDIX A: Supp. Figure 3.2, p. 275) of (*E*)-10-oxooctadec-11-enoic acid (Figure 3.18) indicated a *cis* conjugated double between the olefinic protons H_{11} (δ6.12, dt, *J*= 15.9, 1.4, 1H) and H_{12} (δ6.92, dt, *J* = 15.8, 7.0, 1H), two triplet methylenes (H_2, δ2.26, *J*= 7.5, 2H; H_9, δ2.58, *J*= 7.4, 2H), one triplet methyl (H_{18}, δ0.91, *J*= 6.8, 3H), and unresolved methylenes (δ1.33-2.24; 20 H total). Similar ^{1}H NMR shifts were observed for (*Z*)-10-oxooctadec-11-enoic acid (Figure 3.19; APPENDIX A: Supp. Figure 3.3, p. 276) except for differences in the chemical shifts and coupling constant of the *trans* olefinic protons H_{11} (δ6.23, dt, *J*= 11.5, 1.6, 1H) and H_{12} (δ6.12, dt, *J*= 11.6, 7.5, 1H).

Two-dimensional NMR experiments further elucidated the structures of these fatty acid derivatives. For (*E*)-10-oxooctadec-11-enoic acid, the key ^{1}H-^{13}C HMBC long-range couplings (Figure 3.18, Table 3.13) used to prove the presence of the ketone moiety at C_{10} next to the *cis* double bond at C_{11} to C_{12} were from the olefinic protons H_{11} (δ6.12) and H_{12} (δ6.92) to C_{10} and from the methylene H_9 (δ2.58) to C_{10}. The key correlation that proved the presence of the carboxyl group at C_1 was from the methylene H_2 (δ2.26) to C_1 (δ179.3), C_3 (δ26.4) and C_4 (δ30.0). COSY correlations confirmed the ^{1}H-^{1}H couplings of the much of the structure (Figure 3.18, bold lines). Similar 2D NMR correlations were observed for (*Z*)-10-oxooctadec-11-enoic acid (Figure 3.19 and Table 3.14)

The structures of these fatty acid derivatives were further confirmed through LC-HRMS (Figures 3.20 and 3.21) using negative chemical ionization with a $[M - H]^-$ m/z 295.22720 (calculated for $C_{18}H_{31}O_3^-$, 295.22732, Δ 0.41 ppm) for (*E*)-10-oxooctadec-11-enoic acid and a $[M - H]^-$ m/z 295.22714 (calculated for $C_{18}H_{31}O_3^-$, 295.22732, Δ 0.61 ppm) for (*Z*)-10-oxooctadec-11-enoic acid. Finally, the HRMS-MS experiments (Figure 3.22), using negative chemical ionization, determined the location of the unsaturated ketone system and the number of carbons between the ketone and carboxyl group with the key fragment m/z 199.13397. This HRMS-MS fragmentation data was needed in order to fully solve the structures, as COSY and HMBC correlations were difficult to interpret (due to the overlap of similar chemical shifts) between C_4 to C_7 of both fatty acids.

Neither fatty acid derivative had antimicrobial activity in the bioassays at a concentration of 50 μg ml^{-1}.

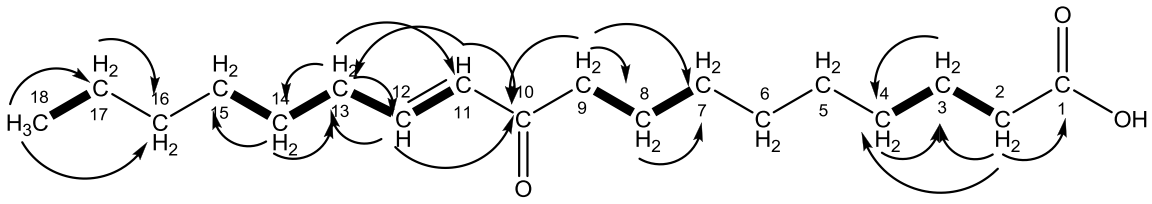


Figure 3.18 Key COSY (bold lines) and HMBC (arrows) correlations for (*E*)-10-oxooctadec-11-enoic acid. HRMS-ESI $[M - H]^-$ m/z 295.22720 (calculated for $C_{18}H_{31}O_3^-$, 295.22732, Δ 0.41 ppm).

Table 3.13 Nuclear magnetic resonance data for (*E*)-10-oxooctadec-11-enoic acid.[a]

Position	**δC, Type**	**δH, mult. (*J* in Hz)**	COSY	HMBC
1	179.3, C	--	--	
2	35.4, CH_2	2.26 t (7.5)	H_3	C_1, C_3, C_4
3	26.4, CH_2	1.58 m	H_2, H_4	C_4
4	30.0, CH_2	1.33 m	H_3	C_3
5,6	30.0, CH_2	1.33 m		
7	30.0, CH_2	1.33 m	H_8	
8	25.2, CH_2	1.59 m	H_7, H_9	C_7
9	40.4, CH_2	2.58 t (7.4)	H_8	C_{10}, C_8, C_7
10	203.9, C	--	--	
11	131.5, CH	6.12 dt (15.9, 1.4)	H_{12}, H_{13}	C_{10}, C_{13}
12	149.3, CH	6.92 dt (15.8, 7.0)	H_{11}, H_{13}	C_{10}, C_{13}
13	33.2, CH_2	2.24 m	H_{11}, H_{12}, H_{14}	C_{11}, C_{12}, C_{14}
14	28.9, CH_2	1.49 m	H_{13}, H_{15}	C_{13}, C_{15}
15	29.8, CH_2	1.35 m	H_{14}	
16	32.4, CH_2	1.32 m		
17	23.4, CH_2	1.33 m	H_{18}	C_{16}
18	14.1, CH_3	0.91 t (6.8)	H_{17}	C_{17}, C_{16}

[a]Measured at 600 MHz (^1H) and 150 MHz (^{13}C) in CD_3OD.
Abbreviations: mult. = multiplicity; Hz = hertz; COSY = correlation spectroscopy; HMBC = heteronuclear multiple bond correlation; m = multiplet; d = doublet; t = triplet; dt = doublet of triplets

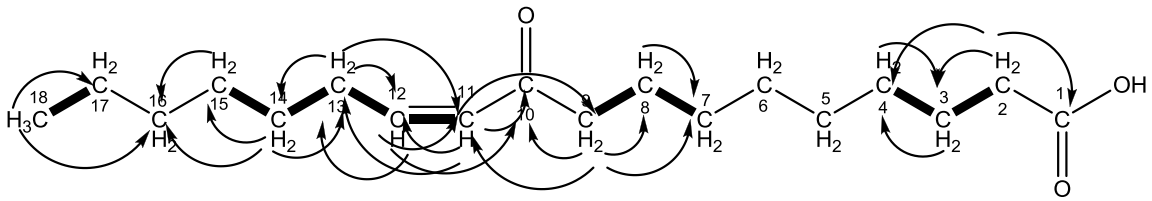


Figure 3.19 Key COSY (bold lines) and HMBC (arrows) correlations for (*Z*)-10-oxooctadec-11-enoic acid. HRMS-ESI $[M - H]^-$ m/z 295.22714 (calculated for $C_{18}H_{31}O_3^-$, 295.22732, Δ 0.61 ppm).

Table 3.14 Nuclear magnetic resonance table of chemical shifts for (*Z*)-10-oxooctadec-11-enoic acid.[a]

Position	**δC**	**δH, mult.**	COSY	HMBC
1	179.0, C	--	--	
2	35.8, CH_2	2.24 t (7.5)	H_3	C_1, C_3, C_4
3	26.4, CH_2	1.59 m	H_2, H_4	C_4
4	30.1, CH_2	1.31 m	H_3	C_3
5,6	30.1, CH_2	1.31 m		
7	30.1, CH_2	1.31 m	H_8	
8	25.0, CH_2	1.57 m	H_7, H_9	C_7
9	44.8, CH_2	2.47 t (7.3)	H_8	C_{10}, C_{11}, C_8, C_7
10	204.5, C	--	--	
11	127.6, CH	6.23 dt (11.5, 1.6)	H_{12}, H_{13}	C_{10}, C_{12}, C_{13}
12	149.5, CH	6.12 dt (11.6, 7.5)	H_{11}, H_{13}	C_{10}, C_{11}, C_{13}
13	30.2, CH_2	2.57 ddd (7.5, 7.5, 1.0)	H_{11}, H_{12}, H_{14}	C_{11}, C_{12}, C_{14}
14	29.9, CH_2	1.44 m	H_{13}, H_{15}	C_{13}, C_{15}, C_{16}
15	30.1, CH_2	1.31 m	H_{14}	C_{16}
16	32.5, CH_2	1.31 m		
17	23.6, CH_2	1.31 m	H_{18}	C_{16}
18	14.2, CH_3	0.90 t (6.9)	H_{17}	C_{16}, C_{17}

[a]Measured at 600 MHz (^1H) and 150 MHz (^{13}C) in CD_3OD.
Abbreviations: mult. = multiplicity; Hz = hertz; COSY = correlation spectroscopy; HMBC = heteronuclear multiple bond correlation; m = multiplet; d = doublet; t = triplet; dt = doublet of triplets; ddd = doublet of doublet of doublet

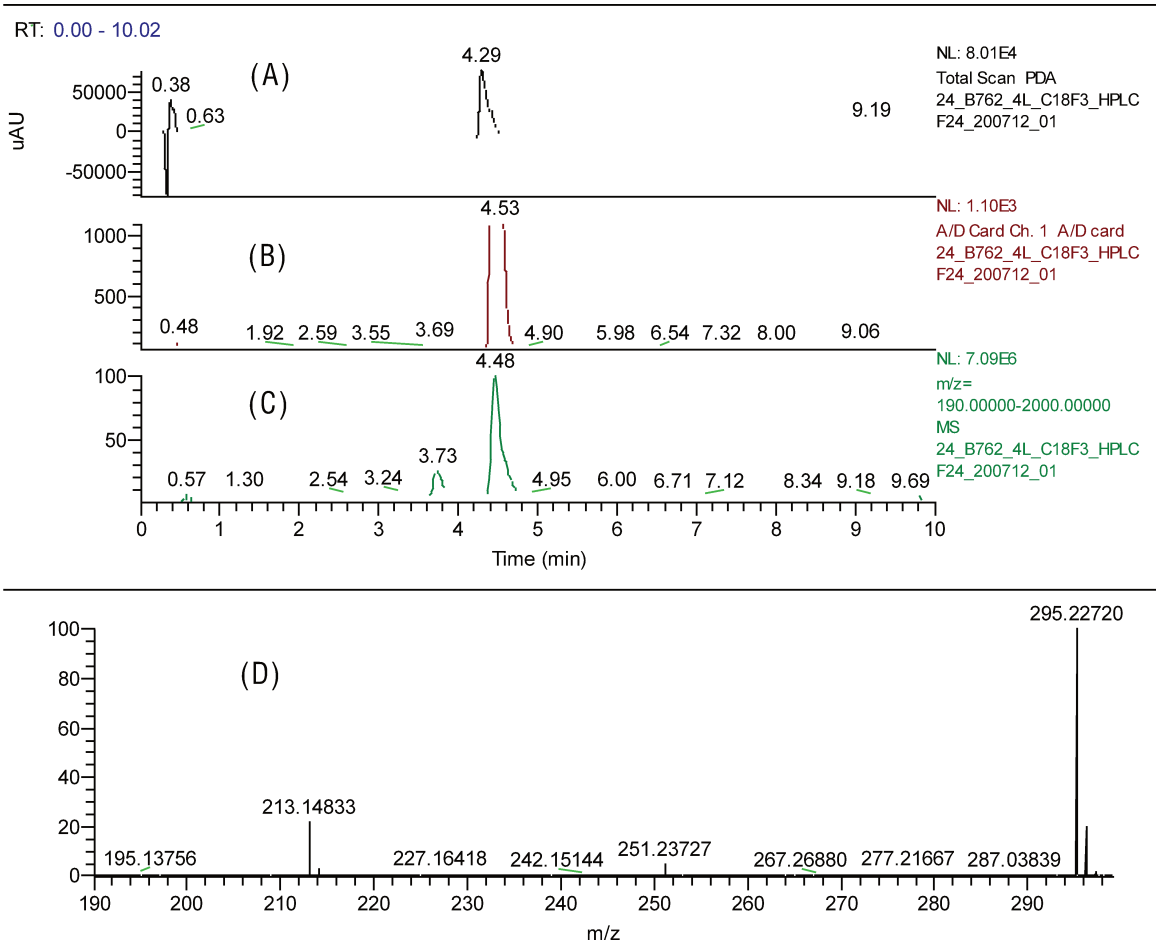


Figure 3.20 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) profile of pure (*E*)-10-oxooctadec-11-enoic acid. (A) UV, (B) Evaporative-light scattering detector (ELSD), and (C) Mass spectrometry (MS) (negative ionization) retention time (RT) profile, as well as the (D) mass spectrum (190.00-300.00 m/z) at a RT of 4.48.

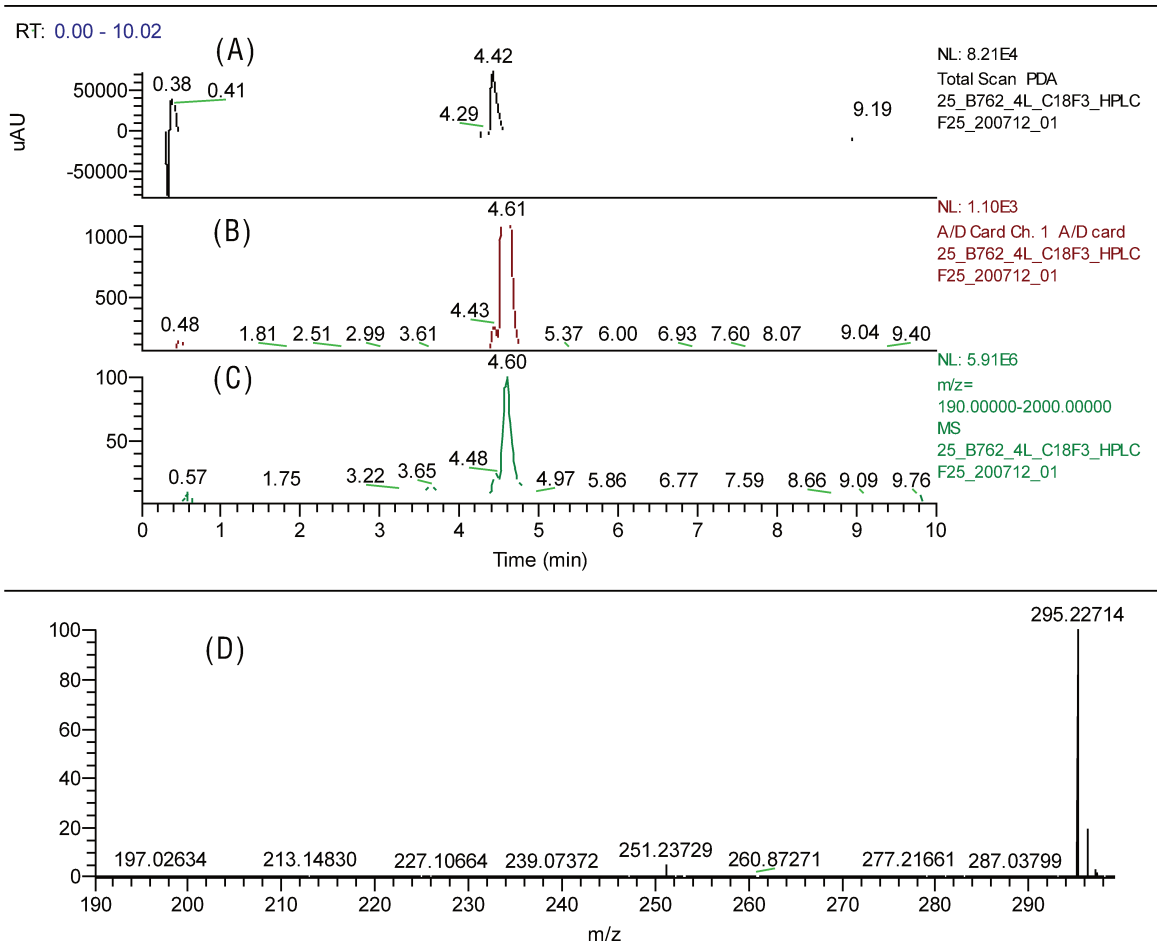


Figure 3.21 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) profile of pure (*Z*)-10-oxooctadec-11-enoic acid. (A) UV, (B) Evaporative-light scattering detector (ELSD), and (C) Mass spectrometry (MS) (negative ionization) retention time (RT) profile, as well as the (D) mass spectrum (190.00-300.00 m/z) at a RT of 4.60.

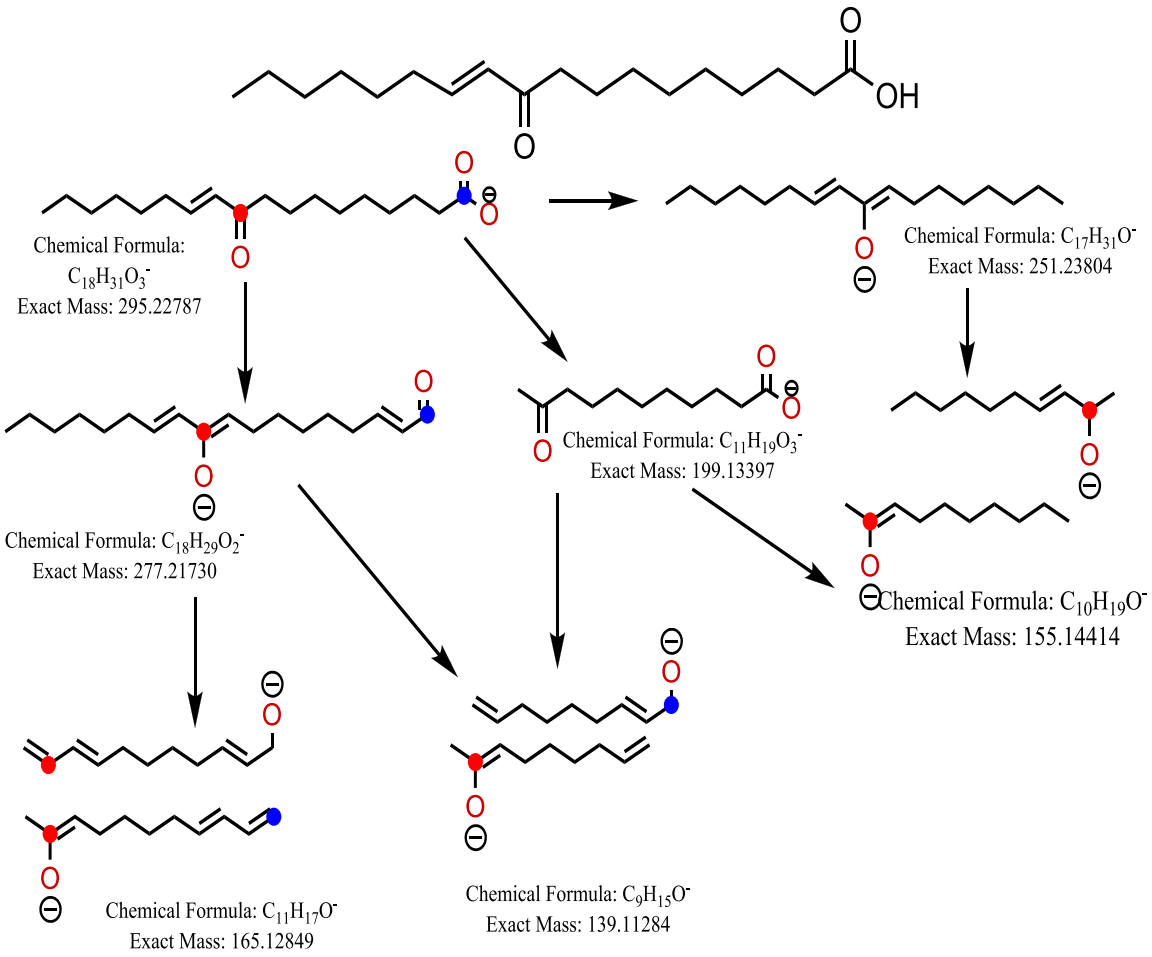


Figure 3.22 High resolution mass spectrometry-mass spectrometry (HRMS-MS) (negative ionization) fragmentation pattern of (*E*)-10-oxooctadec-11-enoic acid. Similar fragmentation was observed for (*Z*)-10-oxooctadec-11-enoic acid (data not shown). Functional groups are shown in red. (-) indicates a negative charge.

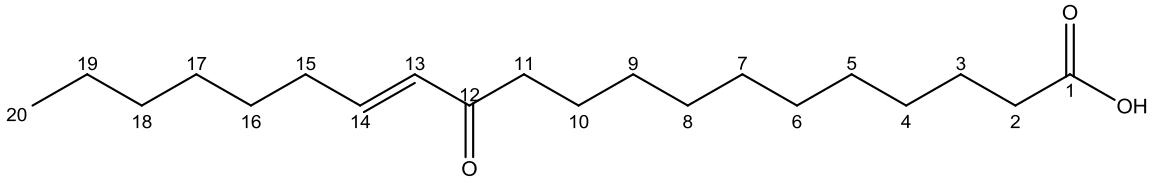
3.3.5.5 The Search for Fuscol and Related Compounds from *Plexauridae*-Associated Microbes

Fuscol is known to exhibit the characteristic ion $[M+H-H_2O]^+$ m/z 271.24160 (calculated for $C_{20}H_{31}^+$, 271.24258, Δ 3.61 ppm) at a RT of 4.84 min. After all of the small-scale *Plexauridae* bacteria fermentation extracts were screened using the XCalibur processing method (see section 3.2.6.4), it was found that the EtOAc extract of the mMB fermentation of *Labrenzia* sp. EF3B-B762 contained a similar ion (m/z 271.24228) to fuscol, eluting at approximately the same RT (4.91 min). Further separation of the extract by C_{18} Sep Pak fractionation revealed that the compound(s) with the characteristic ion and retention time were found in fraction 3. Further HPLC separation of this fraction yielded two additional fatty acid derivatives (both with the molecular formula of $C_{20}H_{36}O_3$), (*E*)-12-oxoicos-13-enoic acid (Figure 3.23.A), with the HRMS-ESI $[M + Na]^+$ m/z 347.25565 (calculated for $C_{20}H_{36}NaO_3^+$, 347.25621, Δ 1.6 ppm), and (*Z*)-12-oxoicos-13-enoic acid (Figure 3.23.B), with the HRMS-ESI $[M + Na]^+$ m/z 347.25546 (calculated for $C_{20}H_{36}NaO_3^+$, 347.25621, Δ 2.2 ppm). Both of these fatty acid derivatives fragmented to give the "fuscol-like" ions in HRMS-ESI $[M+H-H_2O]^+$ m/z 271.24228 (calculated for $C_{20}H_{31}^+$, 271.24258, Δ 1.1 ppm) (Figure 3.26).

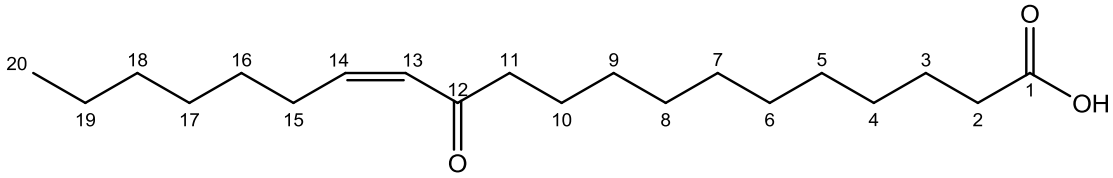
The molecular formulae ($C_{20}H_{36}O_3$) derived from the LC-HRMS data (Figures 3.24 and 3.25) of both compounds suggested that two additional methylene groups were present in the fatty acid chains of these compounds compared to (*E*)- and (*Z*)-10-oxooctadec-11-enoic acid. However, due to the lack of sample material (< 0.5 mg) and the presence of impurities, the full NMR interpretation and chemical shift assignment could not be achieved. Nevertheless, the 1D ^1H (APPENDIX A: Supp. Figure 3.4, p. 277; Supp. Figure 3.5, p. 278) and 2D NMR spectra demonstrated chemical shifts that were similar to (*E*)- and (*Z*)-10-oxooctadec-11-enoic acid, so in combination with the LC-HRMS (Figures 3.24 and 3.25) and HRMS-MS data (Figure 3.26), the structures of these minor fatty acids were proposed to be (*E*)- and (*Z*)-12-oxoicos-13-enoic acid (Figure 3.23). Further HRMS-MS experiments could be run on these compounds in negative

mode in order to deduce the location of the unsaturated ketone moiety and the length of alkyl chain between the ketone and carboxyl moiety, as was done with (*E*)- and (*Z*)-10-oxooctadec-11-enoic acid (Figure 3.22, p. 172). However, the goal of this experiment was simply to prove if the compounds were fuscol, and since they were not, this HRMS-MS experiment was not deemed necessary.

These impure fatty acids did not exhibit antimicrobial activity in the bioassays at the concentration of 50 μg ml^{-1}.



(A)



(B)

Figure 3.23 Predicted structures of *Labrenzia* sp. EF3B-B762 minor, novel fatty acids which fragmented to give the false fuscol production hit. (A) (*E*)-12-oxoicos-13-enoic acid: HRMS-ESI $[M + Na]^+$ m/z 347.25565 (calculated for $C_{20}H_{36}NaO_3^+$, 347.25621, Δ 1.6 ppm). (B) (*Z*)-12-oxoicos-13-enoic acid: HRMS-ESI $[M + Na]^+$ m/z 347.25546 (calculated for $C_{20}H_{36}NaO_3^+$, 347.25621, Δ 2.2 ppm).

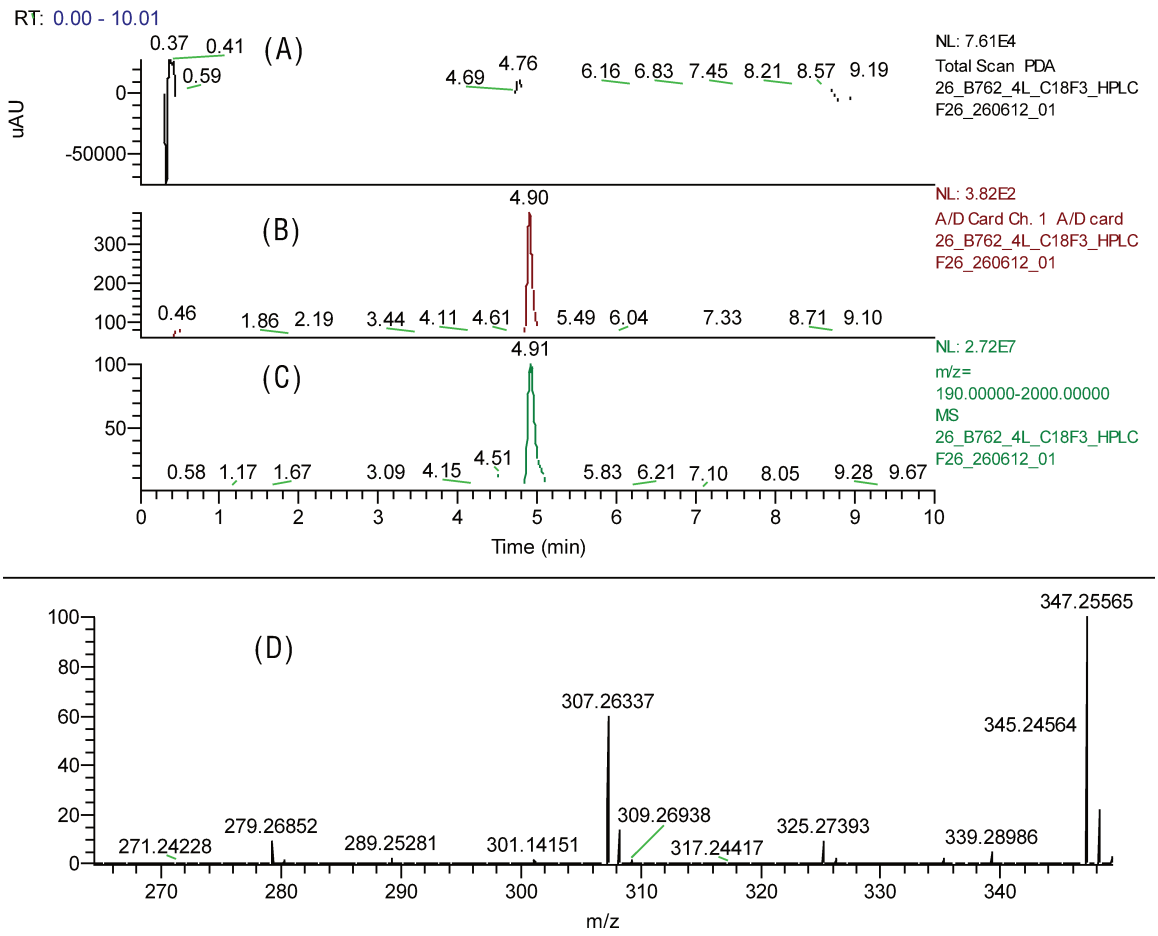


Figure 3.24 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) profile of almost pure (*E*)-12-oxoicos-13-enoic acid. (A) UV, (B) Evaporative-light scattering detector (ELSD), and (C) Mass spectrometry (MS) (positive ionization) retention time (RT) profiles, as well as the (D) mass spectrum (260.00-350.00 m/z) at a RT of 4.91.

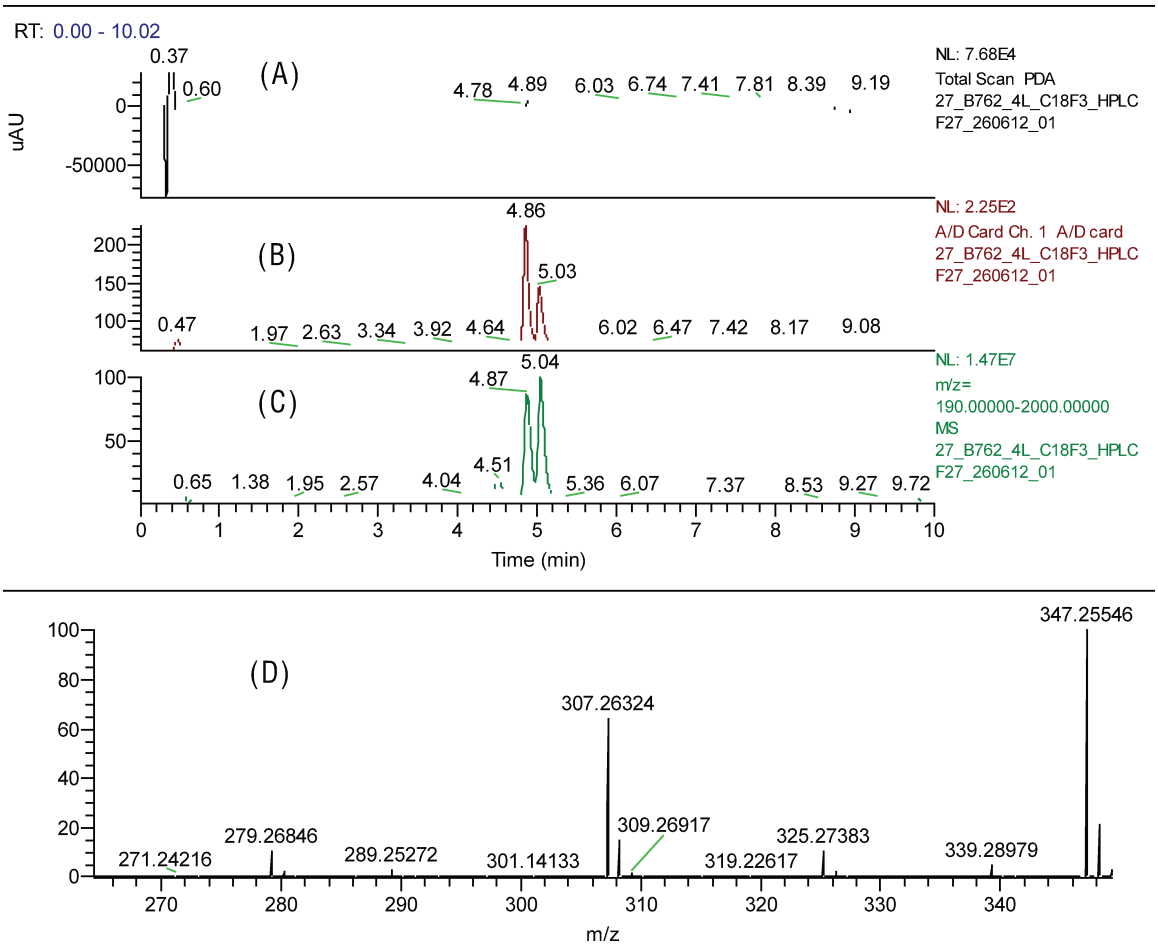


Figure 3.25 Liquid chromatography-high resolution mass spectrometry (LC-HRMS) profile of impure (*Z*)-12-oxoicos-13-enoic acid. (A) UV, (B) Evaporative-light scattering detector (ELSD), and (C) Mass spectrometry (MS) (positive ionization) retention time (RT) profiles, as well as the (D) mass spectrum (270.00-350.00 m/z) at a RT of 5.04.

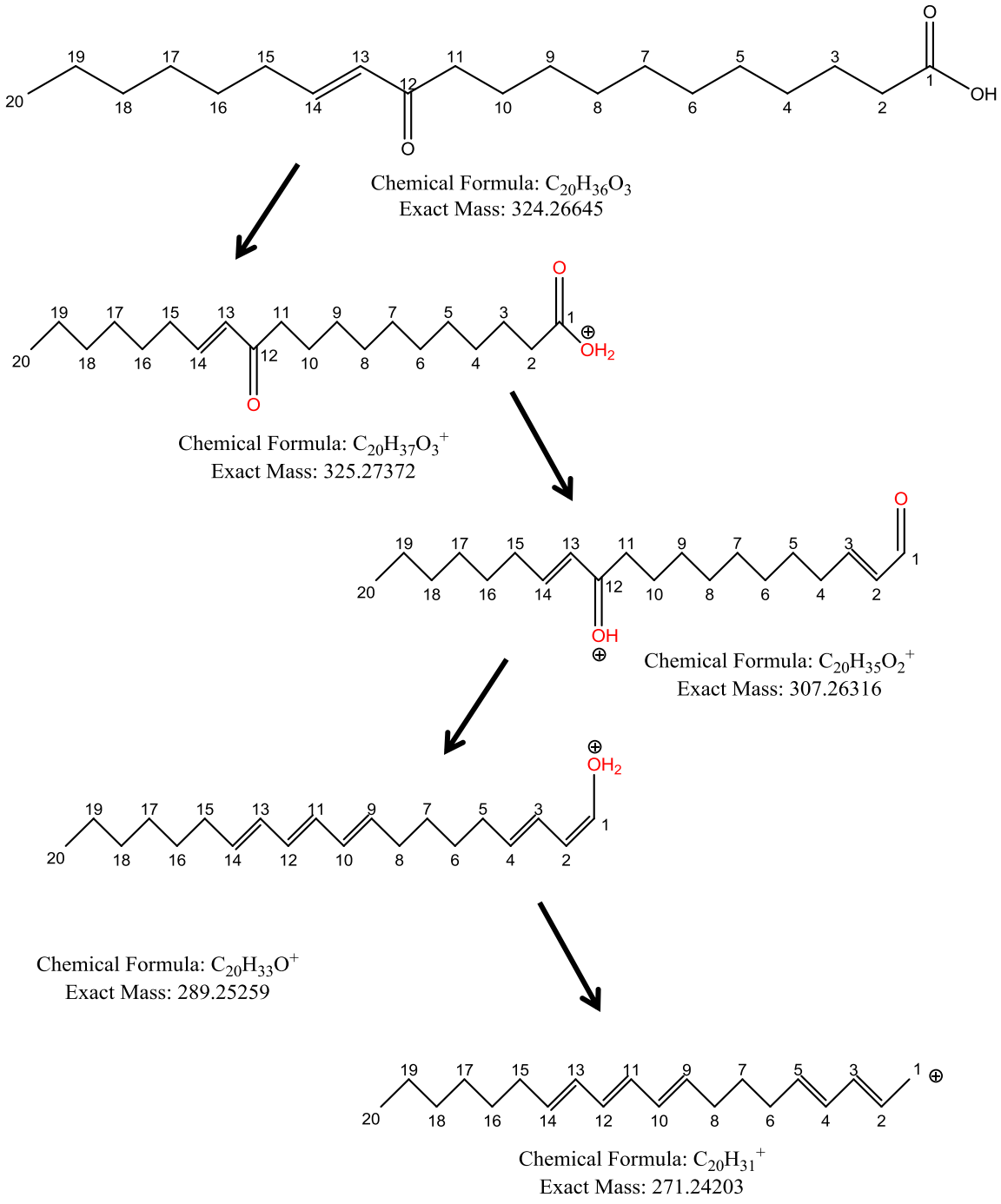


Figure 3.26 High resolution mass spectrometry-mass spectrometry (HRMS-MS) (positive ionization) fragmentation pattern of (*E*)-12-oxoicos-13-enoic acid. Similar fragmentation was observed for (*Z*)-12-oxoicos-13-enoic acid (data not shown). Functional groups are shown in red. (+) indicates a positive charge.

3.4 Discussion

3.4.1 Taxonomic Dereplication of Microbes

In the current study, MALDI-TOF MS[77] was a time- and cost-effective method to dereplicate the bacterial library and decrease the number of identical strains by more than three-fold (1,047 to 330 isolates) prior to 16S rDNA sequencing. After further molecular dereplication, it was found that 137 of the bacteria were unique (*i.e.* <99% 16S rDNA sequence similarity to one another). Because only 41.5% (137/330) of the MALDI-TOF MS deemed "unique bacterial strains" were *molecularly* unique (based on their 16S rDNA sequence), this suggests that either (1) the cut-off for the MALDI-TOF MS dereplication was too stringent and/or (2) the 16S rDNA sequence dereplication was too inclusive.

MALDI-TOF MS dendrogram dereplication at the 20% distance-level was based on the similarity (*i.e.* peak presence and peak intensity) of the mass spectra of the *E. coli* DH15H controls. In support of explanation (1), if some bacterial proteins did not ionize well in some spectra, then this could have caused two bacteria that were in fact the same strain to appear as "different strains" with distance-levels >20%. In addition, there may be greater protein variability in some groups of bacteria, thus, a greater distance-level cut-off may be more accurate for certain groups.

In support of explanation (2), this may be true for bacterial strains with very similar 16S rDNA sequences (*e.g. Vibrio*, *Bacilli*, and *Pseudoalteromonas*). These bacteria may truly be unique strains, as indicated by the MALDI-TOF MS protein analysis, but because their 16S rDNA sequences are ≥99% identical, they were deemed the same strains at the molecular level. If the goal of this project were solely MNP drug discovery, molecular dereplication could hinder downstream novel compound discovery. If this were the case, a complementary natural products dereplication technique, such as LC-HRMS/PCA analysis,[78,79] would be beneficial to explore the potential of similar molecular strains (that were classified as unique by MALDI-TOF MS) to

produce different MNPs. In this study, however, the MALDI-MS dereplication process used was sufficient to identify unique strains for downstream molecular and MNP analyses.

3.4.2 Composition of Cultured Microbial Community from Plexauridae Octocorals

Due to the lack of replicates for non-EF *Plexauridae*, the phylogenetic microbial composition of *E. fusca* (n=9) only will be discussed. The class dominance of *Gammaproteobacteria* in the combined *E. fusca* samples was very similar to other coral-associated bacterial communities.[16,19,21,25,27,29,30,80-86] In addition, the genus-level bacterial composition is also similar to healthy coral libraries previously reported, with the *Gammaproteobacteria* dominated by *Vibrio*[16,19,21,25,27,30,80,82,83,85-89] and *Pseudoalteromonas* spp.,[7,16,17,19,25,76,80,82,83,84,86,88] *Alphaproteobacteria* dominated by *Ruegeria* spp.,[7,25,83] *Bacilli* dominated by *Bacillus* spp.,[24,80,83,85,86] and *Actinobacteria* dominated by *Micrococcus* spp.[24,83,85,86]

Fast-growing, r-selected, heterotrophic bacteria are easy to culture due to their ability to grow on nutrient-rich media at 22-30 ^{o}C, their resilience to antibiotics, and their ability to produce antibiotics.[86,90,91] In this study, the incubation temperature (22-30^{o}C), as well as the nutrient-rich MA medium from which most microbes were isolated, likely contributed to the taxa selection and enrichment of *Vibrio*, *Bacilli*, *Ruegeria*, *Pseudoalteromonas*, and *Micrococcus* cultures.[27,83,92]

The cultured fungi were also similar to isolates previously cultured from marine corals (*Cladosporium* sp.,[93] *Penicillium* sp.,[93] *Didymellaceae* sp.,[94] *Ramularia* sp.,[95] *Exophiala* sp.,[95] *Rhodosporidium* sp.[95], and *Sordariomycetes* sp.[96]) and marine sponges (*Sterigmatomyces* sp.[97]).

3.4.3 Isolation of Novel Microbes from Plexauridae

Even though a large number of frequently isolated, heterotrophic bacteria were cultured, 31 (22.6%) novel, uncharacterized bacteria (<97% 16S rDNA sequence similarity to characterized isolates) were cultured, and this number of novel species would be even greater if a higher 16S rDNA similarity cut-off (*e.g.* 97.5%) were used to define a novel species.[98] This high percentage of novel bacteria is similar to that reported from previous culture-dependent coral studies, where ~32% of cultured bacteria were novel species and one was a novel genus in coral

Fungia scutaria,[83] and 20% were novel species and one was s novel genus in coral *Montastraea franksi*.[80] The isolation of these rare, slow-growing, novel bacteria was made possible through the use of diverse culturing methods. The use of particle-filtration (separating microbes associated with different coral particle sizes), 48-well plates (physically separating microbes in individual), and diverse media (targeting different microbial groups) increased the likelihood of isolating slower-growing microbes.

3.4.4 Microbes to Explore for MNPs in Future Studies

The *Plexauridae* in this study provided an excellent source of unique microbes of high interest for subsequent NP analyses. In addition, many members of the *Plexauridae* cultured library have been previously reported to demonstrate biological activity and/or to possess secondary metabolite biosynthesis genes. The reported biological activity may be through the production of antibiotics or quorum sensing molecules, through changes in nutrient availability from siderophore production and nutrient sequestering, or through alteration of environmental pH.[15] Groups of coral-associated microbes commonly implicated in biological activity included *Vibrio* spp.,[15-19,99,100] *Pseudoalteromonas* spp.,[15-19,76,101,102] *Bacilli* spp.,[17,96] *Halomonas* spp.,[84] *Streptomyces* spp.,[96] *Penicillium* spp.,[96] *Acinetobacter* spp.,[17] *Shewanella* spp.,[17] *Paracoccus* spp.,[17,76] *Marinomonas* spp.,[76] *Pseudovibrio* spp.,[18,19] *Ruegaria* spp.,[15,99] and *Ferrimonas* spp.[18] However, *all* microbes, especially novel species, recovered in the present study represent a promising library to be explored in future studies.

3.4.5 Microbial Isolation Conditions

This culture-dependent study showed that *E. fusca* EF-BS3-B was the most prolific source of microbes (Figure 3.12, p. 152). In comparison to the culture-independent study, however, this sample had a comparable number of OTUs (151) to other EF samples (Table 2.2, p. 45). The majority of EF-BS3-B cultures were *Pseudoalteromonas* spp. and *Vibrio* spp. (Table 3.6, pp. 134-138), groups more likely associated with the seawater than with *Plexauridae* (Table

3.9, pp. 156-157), suggesting that these fast-growing bacteria were not true associates of EF-BS3-B and just passing through the octocoral at the time of collection.

The highest percent of unique microbes was associated with the smaller coral particles (<51 μm) (Figure 3.12, p. 152). Again, this could be because many of these microbes are only passing through the coral and are not true, tissue-associated symbionts. It was also observed that a higher dilution factor increased the yield of unique isolates. Increasing the dilution factor increases the likelihood of isolating slower-growing microbes, explaining why serial dilutions of 10^{-4} are often used in coral culturing studies.[29,89] MA was the most prolific media for the isolation of unique microbes, which has been previously reported in sponge-associated bacterial cultivation.[103] This was likely because certain nutrients (*e.g.* Na^{+}, Cl^{-}, Mg^{2+}) found in the MA formulation are required by marine bacteria, whereas some of the other media lack these essential nutrients and may not support the growth of marine bacteria.

In regards to fungal isolation conditions, undiluted particles yielded the greatest number of fungal cultures. This suggests that there may not be a huge diversity of culturable fungi associated with *Plexauridae*, as the higher dilutions did not contain as many fungal cells. This hypothesis is supported by the decreased diversity (as compared to the bacteria) observed in the fungal DGGE profile of the *Plexauridae* (Chapter 2, Figure 2.8, p. 58). When targeting fungal isolates from the *Plexauridae* in the future, lower dilutions, as well as a greater number of fungal media, such as Glucose Yeast Extract Agar and Potato Dextrose Agar, may increase the chances of isolating a higher diversity of *Plexauridae*–associated fungi.[96,104]

If more than one microbe was isolated under the same condition, it was possible to draw provisional conclusions about certain microbial genera and isolation conditions (Table 3.8, p. 154). These correlations are especially interesting where a large number of microbes (>10) were all isolated under the same, specific conditions. This data can be used in future coral-associated microbial isolation studies to target specific groups of microbes (*e.g.* targeting the isolation of a MNP producing genera), or conversely, preventing the isolation of certain groups of abundantly-

cultured, unfastidious microbes (*e.g. Vibrio* spp.). For an example of the former situation where one might be interested in targeting a specific group of MNP-producing microbes, all *Shewanella* spp. (n=3) were isolated from particles <104 μm, on MA, at The Bahamas site 3, and all *Exophiala* spp. (n=3) were isolated from particles <104 μm - ≥51, at no dilution, on YM, from Florida site 1. Members of these microbial groups have been previously shown be capable of natural product production through genomic[105] or chemical[106] analysis, and thus might be of interest to isolate in future studies. For an example of the latter situation, most *Vibrio* spp. were isolated from a dilution of $1–10^{-1}$ and not on MA, so in order to avoid the isolation of this group, one might increase their dilution factor to 10^{-4} and use MA isolation media.

Although there were a great number of unique and novel isolates associated with certain isolation conditions, it is important to emphasize that all particle sizes, dilutions, and media gave rise to unique microbes. Therefore, the use of a variety of culturing techniques is necessary to increase the probability of isolating unique and novel microbes from *Plexauridae*. In addition, this research validates the use of the particle-filtration technique in a new environment to isolate octocoral, tissue-associated microbes. This method should be considered in future studies, as many unique microbes were isolated on different particle sizes. Moreover, a greater percentage of particle-derived (vs. free-living) bacteria produce bioactive compounds,[15] and therefore, the likelihood of isolating a MNP-producing microbe may increase using this particle-filtration method.

3.4.6 Comparison of Cultured Microbial Community to Culture-Independent Library

Both the culture-dependent and -independent *E. fusca* library were dominated by members of the class *Gammaproteobacteria*. However, the genera composition of this class differed between the studies. The cultured library of *Gammaproteobacteria* was dominated by *Vibrio* spp. and *Pseudoalteromonas* spp., whereas the culture-independent library was dominated by *Endozoicomonas* spp. (Chapter 2). The inability to culture closely-associated bacteria has been previously discussed[80,88,107] and is likely due to the lack of chemical or physical stimuli from

the coral host.[27,82] However, with the diverse isolation conditions used in this study, two novel species of *Endozoicomonas* (EF212^T and PS125^T) were cultured that were nearly identical to members of the *Plexauridae* culture-independent library. This rare isolation provided the opportunity to explore the secondary metabolism of these novel *Endozoicomonas* spp. through antibiosis and fermentation studies, as well as to determine their overall metabolism (Chapter 4), genetic composition and potential functions within the *Plexauridae* (Chapter 5).

Only 0.56% of the total culture-independent *E. fusca* bacterial library (all deprelicated sequences from 454-pyrosequencing and DGGE determined in Chapter 2) was cultured, which is similar to percentages previously reported for culturable versus culture-independent bacterial communities.[88,89] Of particular interest were those bacterial cultures that had ≥99% sequence similarity to a culture-independent sequence and that had highest similarity to a sequence retrieved from the same *Plexauridae* sample in the culture-independent study (*e.g.* *Endozoicomonas* sp. PS125 from PS1-4B). Though rarely reported, the ability to retrieve the same sequence from both libraries provides further evidence in support of a symbiotic relationship of certain groups of bacteria, such as the *Endozoicomonas* spp. with the *Plexauridae*.

Over half of the cultured bacterial sequences were also found in the culture-independent library (*Plexauridae* and seawater samples) at ≥97% sequence similarity between the libraries (Table 3.9, pp. 156-157). Although half of the members of the cultured library had close 16S rDNA relatives in the uncultured library, almost 60% of these sequences were most similar to sequences found in the surrounding water column of the culture-independent library (Table 3.9, pp. 156-157). Thus, even though these water column-associated bacteria may be in low quantities in the *Plexauridae* tissue and are likely not true associates of the *Plexauridae* bacterial community, they may outgrow the more abundant, slower-growing, coral-associated bacteria in a culture-dependent study due to their unfastidious nature. Therefore, both culture-independent and –dependent libraries are necessary to understand the true composition and metabolism of coral-associated microbes.[7]

3.4.7 Investigating Plexauridae-Associated Bacteria for Novel Antimicrobial NPs and Fuscol

3.4.7.1 Antibiosis Study with *Endozoicomonas* spp.

It has been estimated that 20-70% of cultured coral-associated bacteria display antimicrobial activity.[16-18,96,108] These antimicrobials may protect the host coral from invading marine pathogens.[22,23] Specifically, it was shown that bacteria isolated from soft corals produce antibiotics that are active against planktonic bacteria, but not those bacteria associated with the coral.[109]

It was not surprising to see growth inhibition of *Endozoicomonas* EF212^T and PS125^T by a *Bacillus* sp., *Pseudoaltermonas* spp., and a *Streptomyces* sp. (Table 3.10, p. 159), as these bacteria are often reported to produce antimicrobial substances.[15-19,76,96,101,102] However, the interesting morphological changes in the confluence zones between the *Euzebyella* sp. (99% 16S rDNA sequence identity to *Euzebyella saccharophila*[110]) with the *Endozoicomonas* spp. and the *Enterovibrio* sp. (99% 16S rDNA sequence identity to coral pathogen, *Enterovibrio coralii*[111]) with the *Endozoicomonas* spp., were unexpected and thus warranted further examination of the chemical profiles of the *Euzebyella* sp. and *Enterovibrio* sp.

In this study, only *Euzebyella* sp. EF1C-B409 was further examined for its ability to produce novel MNPs, because the isolation of compounds from members of this recently named bacterial genus had not yet been reported, and therefore, the isolation of novel MNPs was anticipated. However, it should be noted that in the future, all bacteria that produced activity against the *Endozoicomonas* spp. (Table 3.10, p. 159) in this antibiosis study should be further examined for their chemical profiles to identify the compounds responsible for the observed bioactivity. In addition, different temperatures should be used in future cross-inhibition studies, as some antimicrobial compounds are temperature sensitive[16,17] and may be less active or completely inactive at the incubation temperature (30^{o}C) used in the present study.

3.4.7.2 Fermentation of *Euzebyella* sp. EF1C-B409, Compound Isolation, Structural Elucidation, and Bioactivity Screening

Fermentation of *Euzebyella* sp. EF1C-B409 yielded the isolation of a novel MNP, 2-isononyl-5-isobutylresorcinol (Figure 3.14, p. 162), which had good Gram-positive bioactivity against MRSA, VRE, and *S. warneri* (Table 3.12, p. 164). Similar 2,5-dialkylresorcinol compounds isolated from *Pseudomonas* spp. exhibited antifungal and antibacterial activity,[112] so it was not surprising to find antimicrobial bioactivity with this novel compound.

The structure of the novel 2-isononyl-5-isobutylresorcinol (Figure 3.14, p. 162) was most closely related to 2-isoundecyl-5-isohexylresorcinol (resorcinin) produced by *Flavobacterium* (previously *Cytophaga*) *johnsoniae*,[113] but the isobutyl side chain on the resorcinol ring was a novel moiety of the 2-isononyl-5-isobutylresorcinol structure (Figure 3.14, p. 162). The novel compound may be an intermediate in flexirubin pigment biosynthesis, a common group of pigments in related bacteria.[114] Flexirubin pigments typically consist of an ω-phenyloctaenic acid chromophore esterified with resorcinol carrying two hydrocarbon chains and are yellow in color, which supports the yellow appearance of *Euzebyella* sp. EF1C-B409. Labeling experiments and genomic analysis suggest that dialkylresorcinol biosynthesis represents a unique offshoot of fatty-acid metabolism in which medium-chain fatty acid precursors are further modified through genes in the *dar* biosynthetic cluster.[112]

Resorcinin was reported to be a mammalian cell growth stimulator of NIH 3T3 mouse fibroblast cells (0.2 to 2 μg ml^{-1}),[113] so it would be interesting to evaluate whether 2-isononyl-5-isobutylresorcinol possessed similar biological activity. If this were the case, the presence of the observed antibacterial and cell growth stimulant biological activities may make this compound an attractive candidate in wound-healing applications.[115]

It was interesting that *Euzebyella* sp. EF1C-B409 demonstrated Gram-positive bioactivity, as the chemical profile of this bacterium was originally explored for its morphological change in the confluence zone with the *Endozoicomonas* spp., Gram-negative bacteria. It is

unclear whether 2-isononyl-5-isobutylresorcinol was involved in the observed rhizoid structure formation (indicative of gliding motility) in the confluence zone with the *Endozoicomonas* spp. Related bacteria have been shown to possess gliding motility,[114] but this motility may be completely unrelated to the bioactive 2-isononyl-5-isobutylresorcinol. This rhizoid development may simply have been the gliding action of the bacterium and not necessarily caused by a direct interaction between the *Euzebyella* sp. and *Endozoicomonas* spp.

As the majority of culturable and culture-independent bacteria from the *Plexauridae* were Gram-negative in origin, it was not surprising to find a bacterium with Gram-positive bioactivity. If a similar antibiosis study were performed with the *Euzebyella* sp. EF1C-B409 as the tester strain, it may inhibit the growth of other Gram-positive bacteria, such as the *Bacilli* spp. or *Streptomyces* sp., cultured from the *Plexauridae* octocorals. In the natural *Plexauridae* environment, the 2-isononyl-5-isobutylresorcinol may prevent the growth of competing, pathogenic Gram-positive bacteria from the surrounding water column.

3.4.7.3 Fermentation of *Endozoicomonas* spp. EF212^T and PS125^T and Bioactivity Screening of Extracts

Although no compounds were produced under the evaluated fermentation conditions, the metabolism (Chapter 4), genetic composition, and potential roles (Chapter 5) for these prominent and ubiquitous members of the *Plexauridae* microbiome are described in subsequent Chapters.

3.4.7.4 Fermentation of *Labrenzia* sp. EF3B-B762, Compound Isolation, Structural Elucidation, and Bioactivity Screening

To date, no MNPs have been reported from *Labrenzia* spp., but a recently sequenced genome of *Labrenzia aggregata* IAM 12614 (J. Craig Venter Institute, NCBI Taxon ID: 384765) demonstrated that this bacterium had a relatively large genome (6.6 megabase pairs) and a high GC content (59.4%), two properties that may suggest the presence of secondary metabolite gene clusters.[116]

The 16S rDNA sequence of *Labrenzia* sp. EF3B-B762 was found in the 454-pyroquencing libraries of five of nine EF samples (EF-FL1-B, C; EF-BS3-B, C; EF-BS4-A), and a greater sequencing depth may have revealed this sequence in all EF. This suggests that this bacterium may play an important role in EF. Moreover, closely-related *Labrenzia* spp. are often associated with dinoflagellates,[118] which may suggest that the *Labrenzia* sp. EF3B-B762 is associated with *E. fusca*-associated clade B1/B184 *Symbiodinium* (Chapter 2). With the presence of the m/z 271.242 ion eluting at the same retention time as fuscol, it was hypothesized that a fuscol-producer had been discovered from an *E. fusca-Symbiodinium*-associated bacterium. However, structural elucidation of the isolated compounds revealed a family of novel fatty acid derivatives (Figure 3.18, p. 168; Figure 3.19, p. 169; Figure 3.23, p. 175), two of which fragmented giving the m/z 271.242 ion (Figure 3.26, p. 178). This family of compounds was previously observed in other *Labrenzia* spp.[118-120] and characterized using gas chromatography (GC) MS,[117] but this is the first time that pure compounds have been isolated and characterized by NMR, LC-HRMS, and HRMS-MS techniques from such bacteria.

Interestingly, many *Labrenzia* spp. have fatty acid profiles dominated by $C_{18:1\omega7}$ (49-71%).[118-120] These compounds are likely components of the bacterial cell wall.[118-121] A biosynthetic pathway has previously been proposed involving lipoxygenase enzymes, allylic rearrangement, and lipohydroperoxidase enzymes for the formation of these compounds from $C_{18:1\omega7}$ precursors.[117]

The most structurally similar compound in Antibase is (*E*)-8-oxo-9-octadecenoic acid (Figure 3.27), isolated from the mushroom, *Clitocybe clavipes*.[122] This compound has reported bioactivity as an inhibitor of the aldehyde dehydrogenase enzyme (IC_{50} = 0.28 mM),[122] so it would be interesting to test this family of novel *Labrenzia* sp. fatty acids for aldehyde dehydrogenase inhibition.

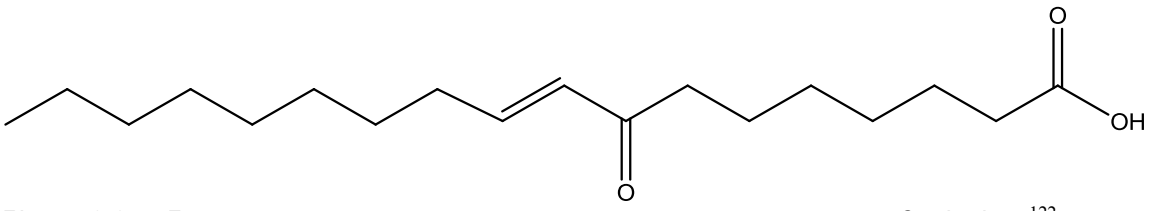


Figure 3.27 (*E*)-8-oxo-9-octadecenoic acid ($C_{18}H_{32}O_3$) isolated from fungi, *C. clavipes*.[122]

3.5 Conclusions

Although less than 1% of coral-associated microbes can be cultured under standard laboratory conditions,[88,89] culture-based studies still provide the best way to access microbial MNPs.[14,123] This study was the first of its kind to thoroughly characterize the culturable microbes of *E. fusca* and related *Plexauridae*.

This study demonstrated that the use of a variety of culturing conditions permitted the isolation of diverse and novel microbes from *Plexauridae*. The use of the particle-filtration technique with a new microbial environment provided an innovative method to isolate microbes associated with differently-sized coral tissue particles and validates this method for future culture-dependent octocoral studies.

This study suggests that *Plexauridae* provide a new source of microbes with the potential to produce novel antimicrobials, as was observed with the isolation of the novel 2,5-dialkylresorcinol compound from *Euzebyella* sp. EF1C-B409. Further studies should investigate the chemical profiles of the numerous MNP producing microbial genera isolated in this study, as well as all of the novel microbes that have never been explored for biological activities. This cultured library thus provides a large resource of untapped and unprecedented biotechnological potential.

Of high interest in the current study was the isolation of two, novel *Endozoicomonas* spp. EF212^T and PS125^T that were also dominant members of the culture-independent library (Chapter 2). This isolation provided the rare opportunity to explore the metabolism of these bacteria through fermentation studies, and even though no compounds of interest were produced under the evaluated fermentation conditions, the metabolism, genomic repertoire, and functions will be further explored in Chapters 4 and 5.

Even though no fuscol producer was found in this study, future studies should continue to explore the *E. fusca* holobiont to determine the true biosynthetic source of this and related diterpenes. A complete understanding of the *E. fusca* microbial community and the ability of an

associated microbe to produce fuscol can only be achieved through the use of culture-independent, culture-dependent, and genomic studies. The culture-independent study is necessary to determine potential stable symbionts within the octocoral; culturing is needed to assess the ability of associated microbes to produce fuscol under different fermentation conditions; and genomic data of cultured isolates is needed to determine the presence of genes encoding for fuscol biosynthesis. A stable-associated microbial symbiont, such as the *Hahellaceae* or *Mycoplasma* relatives discussed in Chapter 2, the associated *Symbiodinium* B1/B184 discussed in Chapter 2, or a yet uncharacterized microbe (*e.g.* an archaea associated with the *Symbiodinium*[57]) may be the true producer, but further studies are needed to confirm these hypotheses.

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CHAPTER 4: FORMAL SPECIES DESCRIPTION: *ENDOZOICOMONAS EUNICEICOLA* SP. NOV. AND *ENDOZOICOMONAS GORGONIICOLA* SP. NOV., BACTERIA ISOLATED FROM THE OCTOCORALS, *EUNICEA FUSCA* AND *PLEXAURA* SP., RESPECTIVELY[a]

[a]Note: This chapter has been peer-reviewed and formally accepted into *IJSEM*. The citation for this manuscript is as follows: Pike, R.E., Haltli, B., & Kerr, R.G. (2013). *Endozoicomonas euniceicola* sp. nov. and *Endozoicomonas gorgoniicola* sp. nov., Bacteria Isolated from the Octocorals, *Eunicea fusca* and *Plexaura* sp. *IJSEM*, doi: 10.1099/ijs.0.051490-0.

4.1 Introduction

The genus *Endozoicomonas*, proposed by Kurahashi and Yokota (2007),[1] is part of the family *Hahellaceae*, order *Oceanospirillales*, class *Gammaproteobacteria*, in the phylum *Proteobacteria*. At the time of carrying out the characterization experiments and writing this species description, there were only two recognized species of *Endozoicomonas*, *E. elysicola* KCTC 12372^T, originally isolated from the sea slug *Elysia ornata*, collected from Izu-Miyake Island, Japan,[1] and *E. montiporae* LMG 24815^T, isolated from the encrusting pore coral *Montipora aequituberculata*, collected from southern Taiwan.[2] More recently, another novel *Endozoicomonas* species was validly published; *E. numazuensis* NBRC 108893^T was isolated from marine purple sponges in the order *Haplosclerida*, collected from a tidal area of Numazu, Japan.[3] This study proposes two novel species of *Endozoicomonas*, EF212^T and PS125^T, isolated from the order *Alcyonacea*, family *Plexauridae* octocorals, *Eunicea fusca* and *Plexaura* sp., collected from Florida, USA, and Bimini, BS, respectively. Table 4.1 summarizes information regarding the isolation source and conditions of all characterized *Endozoicomonas* strains to date.

Table 4.1 Summary of information regarding the isolation of *Endozoicomonas* spp.

Factor	*E. euniceicola* EF212^T	*E. gorgoniicola* PS125^T	*E. montiporae*[2] LMG 24815^T	*E. elysicola*[1] KCTC 12372^T	*E. numazuensis*[3] NBRC 108893^T
Marine Invertebrate Isolation Source	*Eunicea fusca*	*Plexaura* sp.	*Montipora aequituberculata*	*Elysia ornata*	*Haplosclerida* sponges
Location	Florida, USA	Bimini, BS	Southern Taiwan	Izu-Miyake Island, Japan	Numazu, Japan
GPS Coordinates	26°18.068'N, 80°04.112'W	25°31.478'N, 79°17.948'W	N/R	N/R	N/R
Depth (m)	12.5	17.0	10.0-15.0	15.0	N/R
Isolation Media	Difco Marine Agar (MA)	Difco MA	Difco MA	Difco MA	1/10 Difco MA + 75% artificial seawater
Dilution	1/1000	1/1000	Dilution plating	1/10	Direct plating of ground sponge
Particle Size	<50 µm	<50 µm	N/A	N/A	N/A

Abbreviations: N/R = Not Reported; N/A = Not Applicable

4.2 Materials and Methods

4.2.1 Plexauridae Collection, Sample Processing, and Bacteria Isolation

Samples (~30 g) of *E. fusca* (EF-FL2-C) and *Plexaura* sp. (PS1-BS4-B) (see Chapter 2, Table 2.1, p. 34 for explanation of sample ID) were collected by SCUBA diving off the southeastern coast of Florida and Bimini, The Bahamas, respectively, in June of 2009. Healthy samples of octocoral branches were aseptically excised from a coral colony and placed into Whirl-Paks® (Nasco, Fisher Scientific, Toronto, ON) underwater. On the surface, the samples were placed into a bucket of seawater and maintained at 18-22 ^{o}C until samples were processed (< 4 h). Coral samples were aseptically cut into 0.5-1.0 cm lengths, transferred into 50 ml centrifuge tubes, and washed three times with sterile, filtered (0.22 μM Cellulose Acetate, Corning®, VWR, Mississauga, ON) seawater (SFSW) by vigorously shaking to remove loosely-associated bacteria. Approximately half of each octocoral sample was homogenized in SFSW and subsequently separated into different sized particles (≥ 500 μm, ≥ 213 μm, ≥ 104 μm, ≥ 51 μm, and <51 μm) via an adapted-particle filtration apparatus.[4] Serial dilutions of particle sizes 213 - 104 μm, 104 - 51 μm, and < 51 μm were plated onto Marine Agar (MA 2216, BD Difco, VWR, Mississauga, ON) in 48-well plates. Plates were incubated at 22 ^{o}C for up to six months, and strains EF212^{T} and PS125^{T} were purified as single colonies two weeks after initial plating from a 1/1000 dilution of < 51 μm particles plated on MA. Strains were grown in Marine Broth (MB 2216, BD Difco) and preserved in 25% glycerol at -80 ^{o}C.

4.2.2 Obtaining Reference Strains for Comparative Analyses

Reference strains *E. elysicola* KCTC 12372^{T} (=MKT110^{T}) and *E. montiporae* LMG 24815^{T} (=CL-33^{T}) were obtained from the Korean Collection for Type Cultures (KCTC) and Belgian Coordinated Collections of Microorganisms/LMG Bacteria Collection (LMG), respectively, for comparative experiments.

4.2.3 Phenotypic Experiments

4.2.3.1 Cell Morphology and Structure

Cell morphology was determined using a phase-contrast light microscope (Leica DME, EC3 Microsystems, Heerbrugg, Switzerland), and colony morphology was determined with a stereomicroscope (WILD Heerbrugg Leitz Canada Ltd., ON). Cell motility was tested using the hanging drop method,[5] and ultrastructure appendages (*e.g.* flagella and pili) were confirmed using Transmission Electron Microscopy ([TEM], Hitachi BioTEM 7500: Nissei-Sangyo, Rexdale, ON) and imaged using a digital camera (AMT XR40 side mount, Advance Microscopy Techniques, Danvers, MA). For TEM, cells were fixed in 3% glutaraldehyde in sterile Instant Ocean® Sea Salt (36 g l^{-1}, Spectrum Brands, Atlanta, GA), applied to colloidion-coated copper grids (400 mesh), and negatively stained with 5% (w/v) uranyl acetate prepared in 50% (v/v) ethanol. A Gram-stain kit (BD Difco) was used to determine the Gram reaction.

4.2.3.2 Cell Growth Conditions (NaCl, Temperature, pH, and Oxygen)

The NaCl range and optima were tested in Nutrient Broth (NB, EMD Chemicals, VWR) supplemented with increasing concentrations of NaCl (Sigma-Aldrich, Oakville, ON) (0, 0.5, and 1.0 – 5.0% w/v in 1.0% increments). Cultures were grown at room temperature (22 ^{o}C) and 250 rpm for five days. The temperature range and optima was determined in MB 2216 with varying temperatures (4, 15, 22, 30, 37, 45 ^{o}C) at 250 rpm for five days. The pH range and optima were tested in medium composed of 1.0 g l^{-1} peptone (EMD Chemicals, VWR), 5.0 g l^{-1} yeast extract (EMD Chemicals, VWR), and 3.5% NaCl with the following biological buffers: pH 3.0 – 4.0, glycine/HCl buffer; pH 4.0 – 6.0, phosphate-citrate buffer; pH 6.0 – 8.0, Sorensen's phosphate buffer; pH 8.0 – 11.0, glycine/NaOH buffer (all chemicals from VWR or Sigma-Aldrich). Media were prepared at 1.0 pH intervals, and pH was adjusted prior to sterilization and measured post-sterilization to ensure maintenance of a steady pH. Cultures were grown shaking at 250 rpm for five days. All growth experiments were conducted in triplicate, and growth was determined by measuring turbidity (OD_{600}) on a NanoDrop Spectrophotometer (ND-1000, NanoDrop

Technologies, Inc., DE). Anaerobic metabolism was tested using the BD GasPak EZ (BD Difco) kit according to the manufacturer's instructions.

4.2.3.3 Biochemical Characterization Tests (Enzyme Activity and Carbon Utilization)

Strains EF212^T and PS125^T (as well as LMG 24815^T, KCTC 12372^T, and appropriate controls) were phenotypically examined using a number of biochemical characterization tests. Catalase (BD Difco) and oxidase (BD Difco) activity were determined using commercially available kits. DNase (BD Difco) and lipase (BD Difco) activity, as well as the hydrolysis of starch (Fisher Scientific), casein (BD Difco), and Tweens 20, 40, 60, and 80 (Sigma-Aldrich) were determined using standard methods.[6,7] The API 20 NE (bioMerieux, Marcy l'Etoile, France), API ZYM (bioMerieux), and MicroPlate GN2 (Biolog, Hayward, CA) phenotypic tests were used to determine enzymatic activities and carbon source utilization of EF212^T and PS125^T. The API ZYM strips were read after 4.5 h at 30 ^{o}C, the API 20 NE strips were read after 84 h at 30 ^{o}C, and the Biolog GN2 MicroPlates were read after 72 h at 30 ^{o}C. All tests were performed in at least triplicate and according to the manufacturer's instructions, with the exception of the API 20 NE plates, where bacteria were suspended in sterile 2% NaCl (w/v), and the GN2 Biolog plates, where bacteria were suspended in GNGP-IF (per liter: 0.2 g Phytagel, [Sigma-Aldrich] and 0.30 g Pluronic-F68 [Sigma-Aldrich]) with 3% NaCl (w/v).

4.2.3.4 Sensitivity to Antibiotics

Sensitivity to antibiotics was tested by placing discs impregnated with antibiotic (all obtained from Fisher-Scientific) on MA plates inoculated with 100 µl of two-day old cultures of EF212^T and PS125^T (as well as LMG 24815^T) grown in MB (diluted to 0.5 McFarland standard [VWR]). Discs were impregnated with the following quantities of each antibiotic: ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), rifampicin (5 µg), streptomycin (10 µg), tetracycline (30 µg), novobiocin (30 µg), and penicillin G (10 µg). Zones of inhibition indicating antibiotic susceptibility were measured in triplicate

experiments after three days of incubation at 22 °C. Based on previously determined susceptibility/resistance standards,[2] susceptible strains had a zone of inhibition > 3 mm; moderately susceptible strains had inhibition zones 1-3 mm; resistant strains had zones of inhibition < 1 mm.

4.2.4 Chemotaxonomic Experiments

4.2.4.1 Fatty Acid Methyl Ester Content

Analysis of fatty acid methyl esters (FAMEs) was carried out by the Leibniz Institute of Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) (Braunschweig, Germany). Biomass (30 mg freeze dried cells) of strains EF212^T and PS125^T was obtained from cells grown in NB with 3% NaCl at 22 °C, 250 rpm, for 3 days. FAMEs were obtained by saponification, methylation, and extraction using established methods.[8,9] The FAME mixtures were separated using a Sherlock Microbial Identification System (MIS, Version 6.1) (MIDI, Microbial ID, Newark, DE[10]), which consisted of an Agilent model 6890N gas chromatograph fitted with a 5% phenyl-methyl silicone capillary column (0.2 mm x 25 m), a flame ionization detector, Agilent model 7683A automatic sampler, and a HP-computer (Hewlett-Packard Co., Palo Alto, CA) with MIDI database and the methods TSBA40 and TSBA6.

4.2.4.2 Respiratory Quinone Content

Respiratory quinones were estimated using established methods,[11,12] and experiments were carried out by the DSMZ using biomass (200 mg freeze-dried cells) of the two strains prepared as described above.

4.2.4.3 Proteome Analysis Using Mass Spectrometry

Analysis of protein profiles of the two novel strains and LMG 24815^T was carried out using Matrix-Assisted Laser Desorption/Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) (Microflex Bruker Daltonics mass spectrometer, Leipzig, Germany) equipped with a 20.0-Hz nitrogen laser (laser power 50%; up to 400 shots fired; mass range 2,000-12,000 m/z). The MALDI BioTyper Version 2.0 software package (Bruker Daltonik GmbH, Leipzig,

Germany) was used to analyze spectra. Cells grown for 72 h on MA were stamped on a stainless steel target plate overlaid with 1.5 µl matrix (1 ml saturated solution of α-cyano-4-hydroxy-cinnamic acid [Fluka, Sigma-Aldrich] in 50% acetonitrile [Fisher-Scientific] and 2.5% aqueous trifluoroacetic acid [VWR]), allowed to dry, and then examined using MALDI-TOF MS.

4.2.5 Genotypic Experiments

4.2.5.1 DNA G+C Content

The DNA G+C content was carried out by the DSMZ using established methods.[13] Biomass (2 g wet weight) of EF212^T and PS125^T was obtained from cells grown in NB with 3% NaCl at 22^{o}C, 250 rpm, for 3 days.

4.2.5.2 Full-Length 16S rDNA Sequencing

For 16S rDNA analysis, extraction of genomic DNA (gDNA) for both novel strains was carried out using the UltraClean Microbial DNA Isolation Kit (MoBio Laboratories, Inc., VWR) on cells grown in MB for three days, 250 rpm, 30 ^{o}C. PCR amplification of the nearly full-length 16S rRNA gene was done using the universal Eubacteria 16S rRNA primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3')[14] and 1525R (5'AAGGAGGTGATCCAGCC-3')[14] with the following conditions: a 1X concentration of EconoTaq 2X master mix (Lucigen, Middleton, WI), 1.0 µM of each primer, 5% (v/v) DMSO and 40 ng of template DNA. PCR cycling conditions included an initial denaturing period of 3 min at 95 ^{o}C, followed by 35 cycles of 95 ^{o}C for 45 s, 54 ^{o}C for 1 min, and 72 ^{o}C for 1 min, 30 s, and a final extension of 10 min at 72 ^{o}C. A set of five 16S rRNA gene primers (27F; 530R: 5'-GTATTACCGCGGCTGCTG-3'[15]; 514F: 5'-GTGCCAGCASCCGCGG-3'[16]; 936R: 5'-GGGGTTATGCCTGAGCAGTTTG-3'[16]; 1114F: 5'-GCAACGAGCGCAACCC-3'[14]; and 1527R) were used to sequence the nearly full-length 16S rRNA gene amplicons.

Sequencing was performed by Eurofins MWG Operon (Huntsville, AL) and sequences were assembled using Contig Express (Vector NTI Advance 10.3.0, Invitrogen, Carlsbad, CA). The 16S rRNA gene sequences (EF212^T – 1557 bp and PS125^T – 1556 bp) were compared

against available sequences with the Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov),[17] using the BLASTn algorithm to determine approximate phylogenetic relatedness by comparing sequences to those in the GenBank database. In addition, sequences were compared to those in the EzTaxon database[18] (http://eztaxon-e.ezbiocloud.net/). Multiple sequences of closely-related strains were aligned using BioEdit version 7.0.5.3,[19] and phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 4.[20] The evolutionary history of the two novel strains was inferred using multiple methods: Minimum Evolution (ME),[21] Maximum-Parsimony,[22] Unweighted Pair Group Method with Arithmetic Mean,[23] and Neighbor-Joining.[24] A similar topology was obtained in all phylogenetic trees generated on the 43 taxa. Bootstrap analysis was performed with 1000 resamplings.[25] The evolutionary distances were computed using the Jukes-Cantor method[26] and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange algorithm[27] at a search level of 1. The Neighbor-joining algorithm[24] was used to generate the initial tree. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1268 positions in the final dataset.

Although there are only five validly described strains of *Endozoicomonas* to date (including this report), the 16S rRNA gene sequences of several uncultured and cultured bacteria closely related to the characterized strains are present in GenBank. Phylogenetic trees of these closely related isolates were constructed in *MEGA4* as described above. A similar topology was obtained in all phylogenetic trees generated on the 70 taxa. The evolutionary history inferred using the ME method[21] with parameters identical to those described above except that the final dataset consisted of 1189 positions.

4.2.5.3 BOX PCR (Genomic Comparison)

BOX-A1R-based repetitive extragenic palindromic-PCR (BOX PCR) using the BOX1AR primer and established conditions[28] was carried out on EF212^T, PS125^T, KCTC 12372^T, and LMG 24815^T to determine genotypic differences between the closely related type strains.

4.2.5.4 DNA-DNA Hybridization (Genomic Comparison)

To validate the distinct species, DNA-DNA hybridization experiments were performed on EF212^T, PS125^T, KCTC 12372^T, and LMG 24815^T following established methods[29] with previously described modifications.[30] This was carried out by the DSMZ.

4.2.6 16S rDNA Sequence and Strain Accession

Full-length 16S rDNA sequences were deposited in GenBank under the accession numbers JX488684 (EF212^T) and JX488685 (PS125^T). Each strain was also deposited in two recognized culture collections: EF212^T = NCCB 100458^T = DSM 26535^T; PS125^T = NCCB 100438^T = CECT 8353^T.

4.3 Results

Differentiating phenotypic, chemotaxonomic, and genotypic characteristics between the five characterized *Endozoicomonas* species are listed in Table 4.2. A detailed description of all results is given in the formal species descriptions of EF212^T (section *4.4.2*, pp. 225-227) and PS125^T (section *4.4.3*, pp. 227-228).

4.3.1 Phenotypic Experiments

Figure 4.1 and Figure 4.2 show TEM images of EF212^T and PS125^T ultrastructure appendages, respectively. Carbon source utilization is provided in Table 4.3.

Table 4.2 Differential phenotypic, chemotaxonomic, and genotypic characteristics of *Endozoicomonas* spp.

Characteristic	*E. euniceicola* EF212^T	*E. gorgoniicola* PS125^T	*E. montiporae*[2] LMG 24815^T	*E. elysicola*[1] KCTC 12372^T	*E. numazuensis*[3] NBRC 108893^T
Motility	motile	motile	motile	motile	non-motile
Cell length, diameter (μm)	0.6-0.9, 1.7-2.6	0.4-0.9, 1.7-2.5	0.5-0.7, 1.0-3.0	0.4-0.6, 1.8-2.2	3.0-10.0, 0.4-0.8
Colony color	white	creamy white	beige	transparent-white	pale creamy white
Colony diameter (mm)	0.2-0.5 mm	0.5-1.0 mm	1.0-2.0 mm	4.0-5.0 mm	1.5-2.0 mm
Temp. growth range	15-30 °C	15-30 °C	15-35 °C	4-37 °C	15-37 °C
Temp. growth optima	22-30 °C	22-30 °C	25 °C	25-30 °C	25 °C
pH growth range	7.0-8.0	7.0-9.0	6.0-10.0	ND	5.5-9.0
pH growth optima	8.0	8.0	8.0	ND	7.5-8.0
NaCl growth range	1-4%	1-4%	1-3%	no growth w/o NaCl	1-5%
NaCl growth optima	2-3%	2-3%	2-3%	no growth w/o NaCl	2%
Aerobe/Anaerobe	Facultative anaerobe	Facultative anaerobe	Aerobe	Aerobe	Facultative Anaerobe
API ZYM/20 NE:					
Nitrate reduction	–	–	+	+	+
Esculin hydrolysis (β-glucosidase)	W	W	+	+	–
Gelatin hydrolysis (protease)	–	–	–	–	V
β-galactosidase	W	W	W[#]	–	–
Esterase Lipase (C8)	+	+	+	–	+
N-acetyl-β-glucosaminidase	–	–	–	+	–
Biolog Carbon Sources:					
Dextrin	V	–	+	–[2]	–
D-arabitol	–	V	–	–[2]	–
D-galactose	–	–	+	+[2]	–
α-D-lactose	V	+	+	+[2]	–
Maltose	V	+	+	+[2]	–
D-mannose	–	+	–	–[2]	+
Glycerol	V	+	+	–[2]	+
Antibiotics:					
Gentamicin (10 μg)	S	MS	S	ND	ND
Tetracycline (30 μg)	MS	S	MS[#]	ND	ND
Major fatty acids	16:1ω6c/ 16:1ω7c; $C_{16:0}$; 18:1ω7c; $C_{14:0}$	16:1ω6c/16:1ω7c; 18:1ω7c; $C_{16:0}$; $C_{14:0}$	18:1ω7c[#]; 16:1ω6c/16:1ω7c; $C_{16:0}$; $C_{14:0}$	16:1ω6c/ 16:1ω7c; $C_{16:0}$; 18:1ω7c; $C_{14:0}$	18:1ω7c; 16:1ω7c; $C_{16:0}$
Major respiratory quinones	Q9 & Q8	Q9 & Q8	Q9 & Q8	Q9 & Q8	Q9 & MK9
G+C content (mol %)	48.6	47.5	48.9[#]	50.4	48.7
Isolation source (*Order*)	*Eunicea fusca* (*Alcyonacea*)	*Plexaura sp.* (*Alcyonacea*)	*Montipora aequituberculata* (*Scleractinia*)	*Elysia ornata* (*Sacoglossa*)	Marine sponges (*Haplosclerida*)

All experiments in this study performed at least in triplicate. [#]Data obtained in current study.
Abbreviations: (+) = Positive result; (−) = Negative result; W = Weak result; V = Variable results; ND = Not determined; S = Susceptible (>3 mm inhibition zone); MS = Moderately susceptible (1-3 mm inhibition zone)

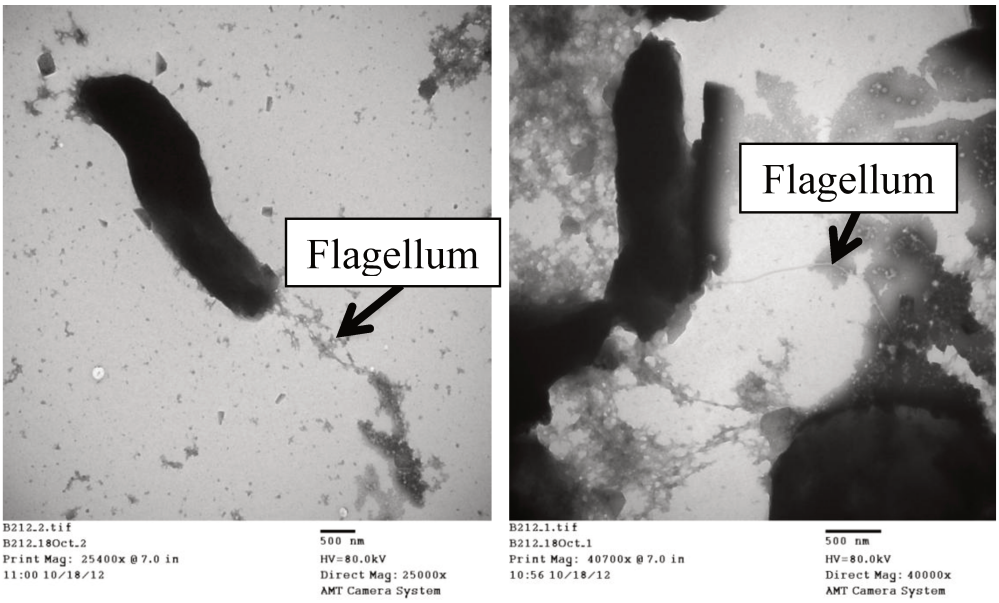


Figure 4.1 Transmission electron microscopy (TEM) images of EF212^{T}. Ultrastructure appendage (flagellum) was confirmed using TEM.

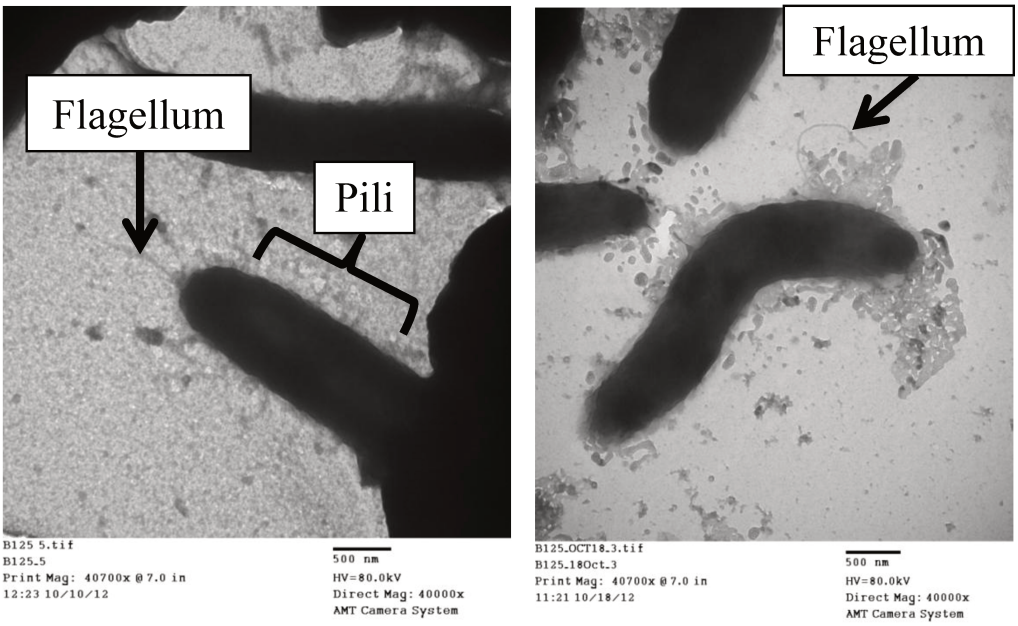


Figure 4.2 Transmission Electron Microscopy (TEM) images of PS125^{T}. Ultrastructure appendages (flagellum and pili) were confirmed using TEM.

Table 4.3 Results of Biolog GN2 MicroPlates. Carbon source utilization differences between *Endozoicomonas* spp.

Carbon Source	*E. euniceicola* EF212^T	*E. gorgoniicola* PS125^T	*E. montiporae*[2] LMG 24815^T	*E. elysicola*[2] KCTC 12372^T	*E. numazuensis*[3] NBRC 108893^T
α-cyclodextrin	–	+	–	–	ND
dextrin	V	–	+	–	–
glycogen	–	V	–	–	ND
Tween 40	+	+	+	W	ND
Tween 80	+	+	+	W	ND
N-acetyl-D-galactosamine	–	V	–	–	ND
N-acetyl-D-glucosamine	+	+	+	+	+
D-arabitol	–	V	–	–	–
D-cellobiose	–	–	W	–	–
i-erythritol	–	V	–	–	ND
D-fructose	–	–	–	–	V
L-fucose	–	–	–	+	ND
D-galactose	–	–	+	+	–
α-D-glucose	+	+	+	+	+
m-inositol	–	V	–	–	–
α-D-lactose	V	+	+	+	–
lactulose	–	+	–	–	ND
maltose	V	+	+	+	–
D-mannitol	–	V	–	–	–
D-mannose	–	+	–	–	+
D-melibiose	–	V	W	–	ND
D-raffinose	–	+	–	–	ND
L-rhamnose	–	V	–	–	–
D-sorbitol	–	V	–	–	–
D-trehalose	–	V	–	–	–
turanose	–	V	–	–	ND
xylitol	–	V	–	–	–
pyruvic acid methyl ester	–	–	+	–	ND
succinic acid mono-methyl-ester	V	–	W	–	ND
acetic acid	V	V	W	–	ND
citric acid	–	V	–	+	ND
D-gluconic acid	–	V	–	–	–
D-glucosaminic acid	–	V	–	–	ND
β-hydroxybutyric acid	–	V	–	–	ND
γ-hydroxybutyric acid	–	V	–	–	ND
α-keto butyric acid	–	–	+	+	ND
α-keto glutaric acid	+	+	+	+	ND
D,L-lactic acid	–	V	–	–	ND

malonic acid	−	V	−	−	ND
propionic acid	−	−	W	−	ND
quinic acid	−	V	−	−	ND
D-saccharic acid	−	V	−	−	ND
succinic acid	+	+	+	−	ND
bromosuccinic acid	V	V	+	−	ND
succininamic acid	−	V	−	−	ND
L-alaninamide	+	+	+	−	ND
L-alanine	+	+	+	−	ND
L-alanyl-glycine	+	+	−	−	ND
L-asparagine	V	+	−	−	ND
L-aspartic acid	V	+	−	−	ND
L-glutamic acid	−	+	−	−	ND
glycyl-L-aspartic acid	−	+	−	−	ND
glycyl-L-glutamic acid	V	+	−	−	ND
L-histidine	−	V	−	−	ND
L-ornithine	−	V	−	−	ND
L-proline	+	+	−	−	ND
L-threonine	+	−	−	−	ND
γ-amino butyric acid	−	+	−	−	ND
inosine	V	+	W	−	ND
uridine	V	+	+	−	ND
thymidine	−	−	+	−	ND
putrescine	−	V	−	−	ND
2-aminoethanol	−	+	−	−	ND
2,3-butanediol	−	V	−	−	ND
glycerol	V	+	+	−	+
D,L-α-glycerol phosphate	−	+	−	−	ND
α-D-glucose-1-phosphate	−	+	−	−	ND

Carbon sources not reported in table if negative result (or ND) for all strains. All carbon sources in this study were tested at least in triplicate experiments. [#]Data obtained in current study.
Abbreviations: (+) = Positive result; (−) = Negative result; V = Variable result; W = Weak result; ND = Not determined (or not reported)

4.3.2 Chemotaxonomic Experiments

4.3.2.1 Fatty Acid Methyl Ester Content

The predominant fatty acids of EF212^T and PS125^T were summed feature 3 ($C_{16:1}\omega6c$ and $C_{16:1}\omega7c$), summed feature 8 ($C_{18:1}\omega7c$), $C_{16:0}$, and $C_{14:0}$. A comparison of the five, characterized *Endozoicomonas* strain FAME profiles is shown in Table 4.4.

4.3.2.2 Respiratory Quinone Content

The major respiratory quinones of EF212^T and PS125^T were ubiquinone Q-9 (79% and 72%, respectively) and Q-8 (22% and 21%, respectively). A comparison of the respiratory quinones between the five, characterized *Endozoicomonas* strains is provided in Table 4.5.

4.3.2.3 Protein Content

Differences in protein profiles were observed between EF212^T, PS125^T, and 24815^T (Figure 4.3).

Table 4.4 Cellular fatty acid methyl ester composition of *Endozoicomonas* spp.

	E. euniceicola EF212^T	*E. gorgoniicola* PS125^T	*E. montiporae*[2] LMG 24815^T		*E. elysicola*[1] KCTC 12372^T	*E. numazuensis*[3] NBRC 108893^T
Hydroxy:			Reported[2]	Observed (DSMZ)[#]	Reported[1]	Reported[3]
3-OH $C_{10:0}$	3.0	2.4	2.9	3.0	3.1	4.9
3-OH $C_{12:0}$	1.0	0.6	–	0.5	2.8	2.7
3-OH $C_{12:1}$	–	–	–	–	–	-
3-OH $C_{14:0}$	–	–	–	–	4.1	-
3-OH $C_{16:0}$	–	–	–	–	–	0.6
Saturated:						
$C_{10:0}$	–	–	–	–	1.0	–
$C_{12:0}$	–	0.5	–	–	6.5	–
$C_{14:0}$	13.8	8.5	8.5	6.8	9.3	0.9
$C_{15:0}$	–	–	–	–	0.7	–
$C_{16:0}$	17.1	17.1	12.0	14.6	18.9	20.0
$C_{18:0}$	0.5	–	–	0.5	0.8	3.1
$C_{19:0}$	–	–	–	–	–	0.7
Summed Features:						
2 (16:1 iso I/ 14:0 3OH)	2.8	1.0	1.5	1.6	–	1.8
3 (16:1ω6c/ 16:1ω7c)	44.0	49.4	39.6	31.4	54.5 (16:1ω7c)	31.5 (16:1ω7c)
8 (18:1ω7c/ 18:1ω6c)	15.97 (18:1ω7c)	19.45 (18:1ω7c)	32.8	41.0 (18:1ω7c)	5.5 (18:1ω7c)	32.9 (18:1ω7c)

Fatty acid composition reported as percentages. Analysis carried out in duplicate experiments by the DSMZ, Germany. [#]Data obtained in current study. Abbreviation: (−) = <0.5% or not detected in strain

Table 4.5 Isoprenoid quinones of *Endozoicomonas* spp.

	E. euniceicola EF212^T	*E. gorgoniicola* PS125^T	*E. montiporae*[2] LMG 24815^T	*E. elysicola*[1] KCTC 12372^T	*E. numazuensis*[3] NBRC 108893^T
Q8 Ubiquinone	22	21	5; 19[#]	18	6
Q9 Ubiquinone	79	72	91; 66[#]	81	61
MK-8	–	–	–	–	4
MK-9	–	–	–	–	29
Unknown	trace	trace	4; 14[#]	–	–

Respiratory quinone composition reported as percentages. Analysis carried out by DSMZ, Germany. [#]Data obtained in current study. Abbreviation: (−) = <0.5% or not detected in strain

(A)

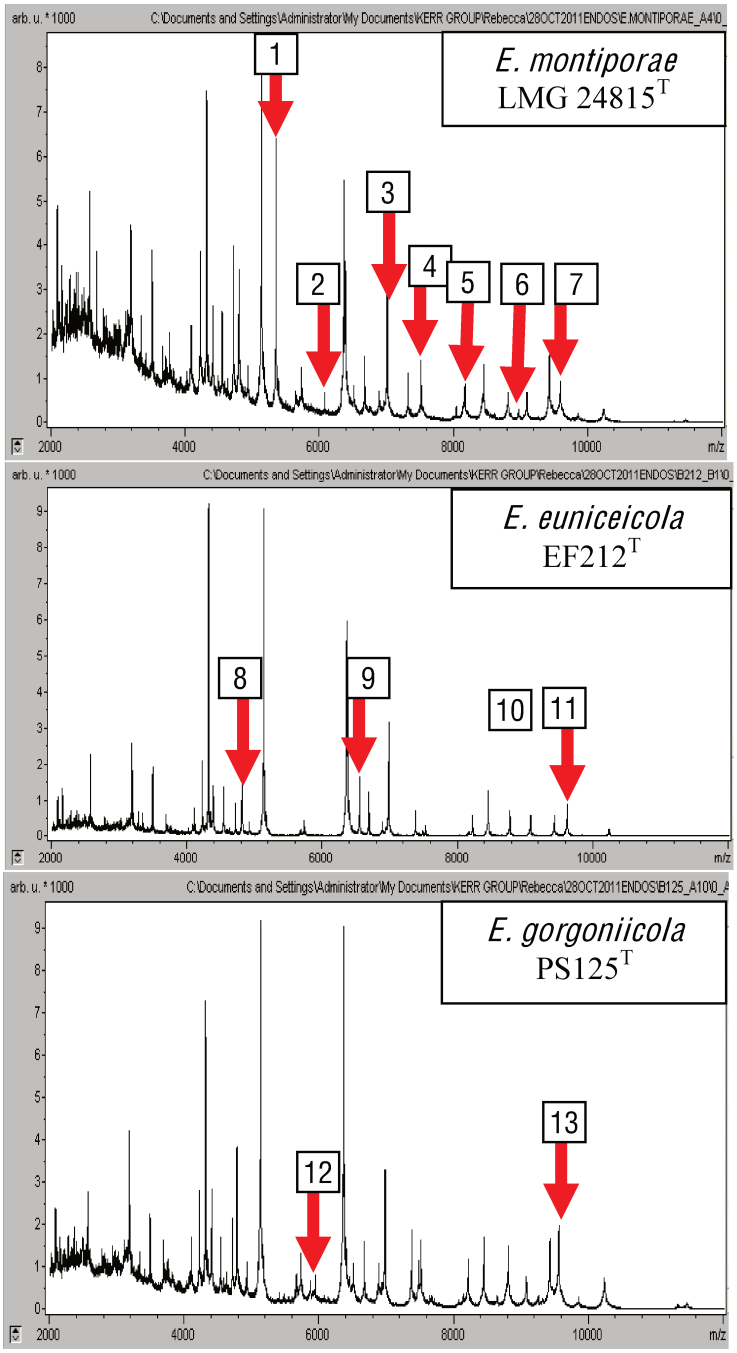
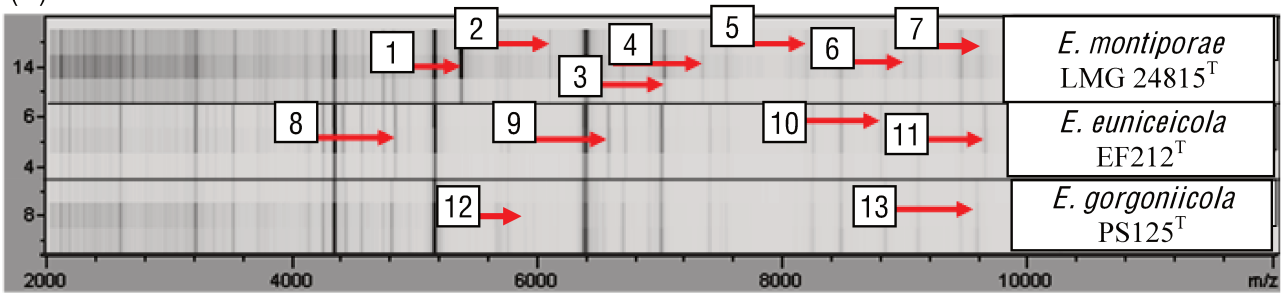


Figure 4.3 Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI-TOF MS) spectra of *Endozoicomonas* spp. Chemotaxonomic evidence of different protein production in the individual species indicated with red arrows. MALDI-TOF MS experiment was performed in triplicate on three separate samples to ensure reproducibility of protein fingerprints. (A) Mass spectra protein fingerprints, 2,000-12,000 m/z. (B) Top view of mass spectra to better compare differences between protein fingerprints. Red arrows highlight different proteins between spectra, and numbers correspond to different protein peaks in (A) and (B) views of the mass spectra.

(B)



4.3.3 Genotypic Experiments

4.3.3.1 DNA G+C Content

The DNA G+C content of strains EF212^T and PS125^T was 48.6 and 47.5 mol%, respectively.

4.3.3.2 Full-Length 16S rDNA Analysis

A comparison of the nearly full-length 16S rRNA gene sequence of strains EF212^T and PS125^T to members of closely-related genera showed that the novel strains formed a clade with the closely-related strains, *E. montiporae* LMG 24815^T, *E. elysicola* KCTC 12372^T, and *E. numazuensis* NBRC 108893^T, comprising the genus *Endozoicomonas* (Figure 4.4, red box) in the class *Hahellaceae* (Figure 4.4, blue box).

Using pairwise sequence comparisons, strains EF212^T and PS125^T had 16S rDNA sequence similarity values ≤ 97% (the commonly used cut-off level to define a different species) to all other strains and to each other. The 16S rDNA sequence similarity values for EF212^T and PS125^T were as follows: to *Endozoicomonas montiporae* LMG 24815^T, 97.2% and 95.6%, respectively; to *E. elysicola* KCTC 12372^T, 95.6% and 96.4%, respectively; to *E. numazuensis* NBRC 108893^T, 96.1% and 95.1%, respectively; to each other, 94.6%. The 16S rRNA gene sequence similarity between EF212^T and PS125^T and other validly described species of bacteria within the family *Hahellaceae* and order *Oceanospirillales* of the *Gammaproteobacteria* were below 94% and 95%, respectively.

When comparing the 16S rDNA of closely-related clones and isolates (Figure 4.5), the *Endozoicomonas* phylotypes loosely grouped by isolation source (*e.g.* octocoral, coral, sponge, bivalve), which is supported by EF212^T grouping within an octocoral clade. However, PS125^T grouped within a sponge and coral clade.

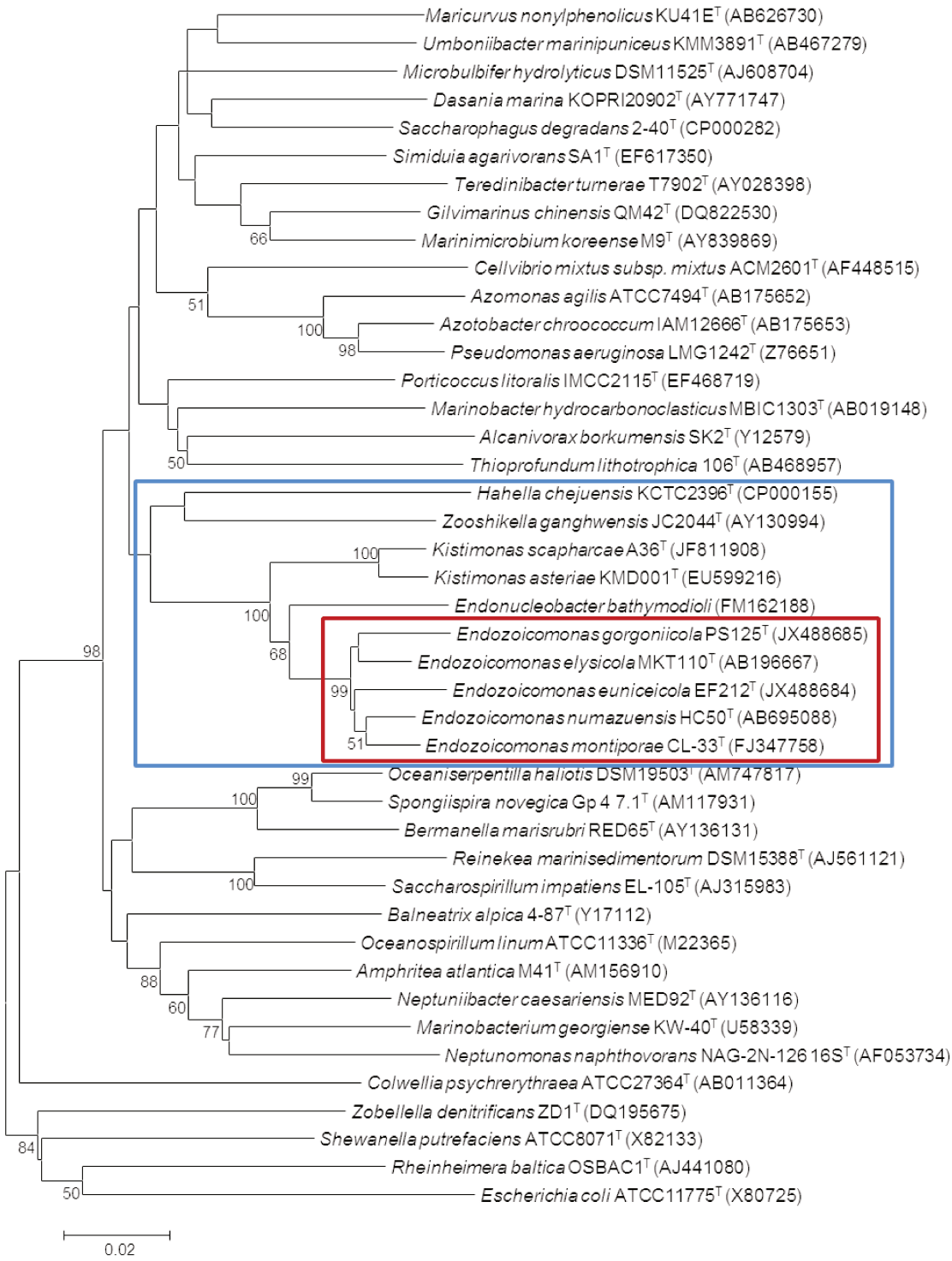
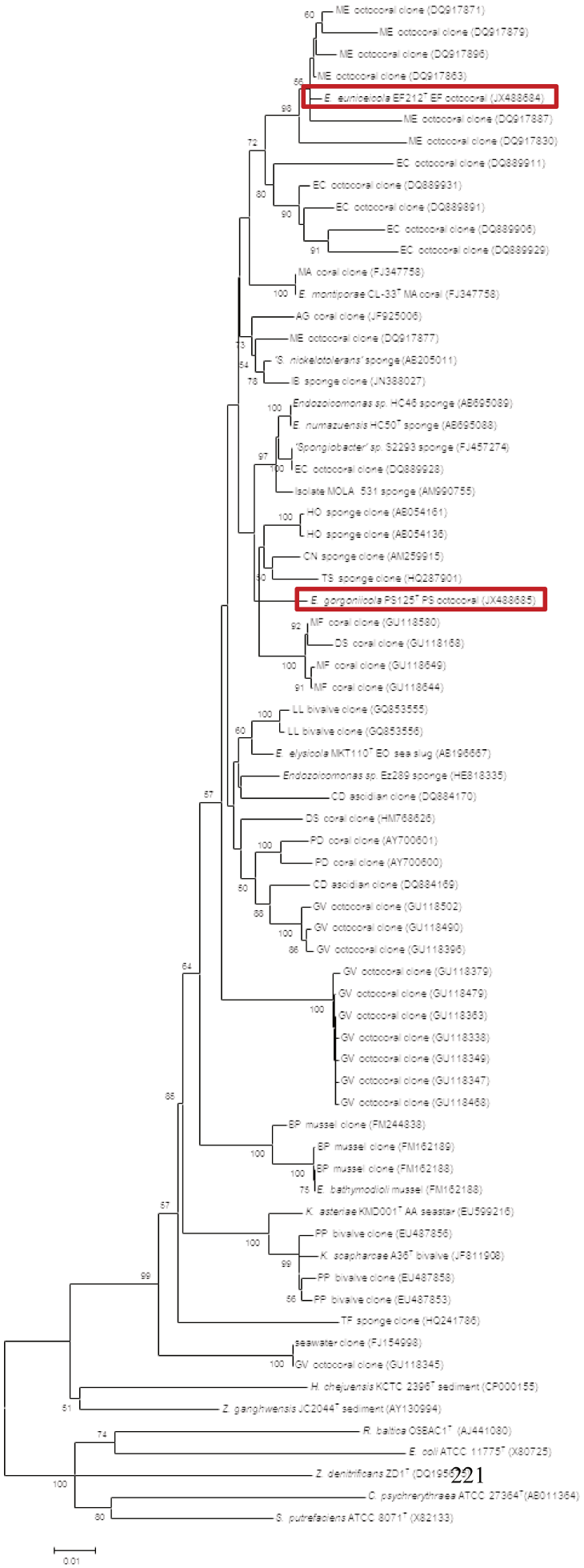


Figure 4.4 Phylogenetic relationships of 43 type strains based on 16S rRNA gene sequences. The evolutionary history was inferred using the Minimum Evolutionary method. The Neighbor Joining algorithm was used to construct the tree based on a comparison of 1268 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch points; bootstrap values <50% are not shown. The Bar length represents 0.02 substitutions per nucleotide position. Red box indicates genus *Endozoicomonas*; blue box indicates family *Hahellaceae*. *E. coli* ATCC11775^T was used as the outgroup to root the tree.



Octocorals:
EC = *Erythropodium caribaeorum*
EF = *Eunicea fusca*
GV = *Gorgonia ventalina*
ME= *Muricea elongata*
PS = *Plexaura* sp.
AG = *Alcyonium gracillimum*
Hexacorals (hard corals):
DS = *Diploria strigosa*
MF = *Montastraea faveolata*
MA = *Montipora aequituberculata*
PD = *Pocillopora damicornis*
Sponges:
CD = *Chondrilla nucula*
HO = *Halichondria okadai*
IB = *Ianthella basta*
TF = *Tsitsikamma favus*
TS = *Tsitsikamma* sp.
Ascidian:
CD = *Cystodytes dellechiajei*
Sea star:
AA = *Asterias amurensis*
Gastropods:
EO = *Elysia ornata*
Bivalve:
BP = *Bathymodiolus puteoserpentis*
LL = *Loripes lacteus*
PP = *Phacoides*

Figure 4.5 Phylogenetic relationships of 70 *Endozoicomonas* spp. related clones and pure cultures constructed from 16S rRNA gene sequences. The evolutionary history was inferred using the Minimum Evolutionary method. The Neighbor Joining algorithm was used to construct the tree based on a comparison of 1189 nucleotide positions. Bootstrap values are expressed as percentages of 1000 replicates at the branch points; bootstrap values <50% are not shown. The Bar length represents 0.01 substitutions per nucleotide position. Red boxes indicate *Endozoicomonas* spp. nov. described in this report. *E. coli* ATCC11775^{T} was used as the outgroup to root the tree.

4.3.3.3 BOX PCR (Genomic Comparison)

Significant differences in BOX PCR fingerprinting patterns (Figure 4.6) were observed between all strains examined indicating each bacterium represents a distinct strain of *Endozoicomonas*, further confirming the same conclusion drawn from comparison of 16S rDNA sequences.[31,32]

4.3.3.4 DNA-DNA Hybridization (Genomic Comparison)

The results of this experiment confirmed four distinct species with DNA-DNA relatedness values well below the 70% cut-off level commonly used to define a genomic species (Table 4.6).

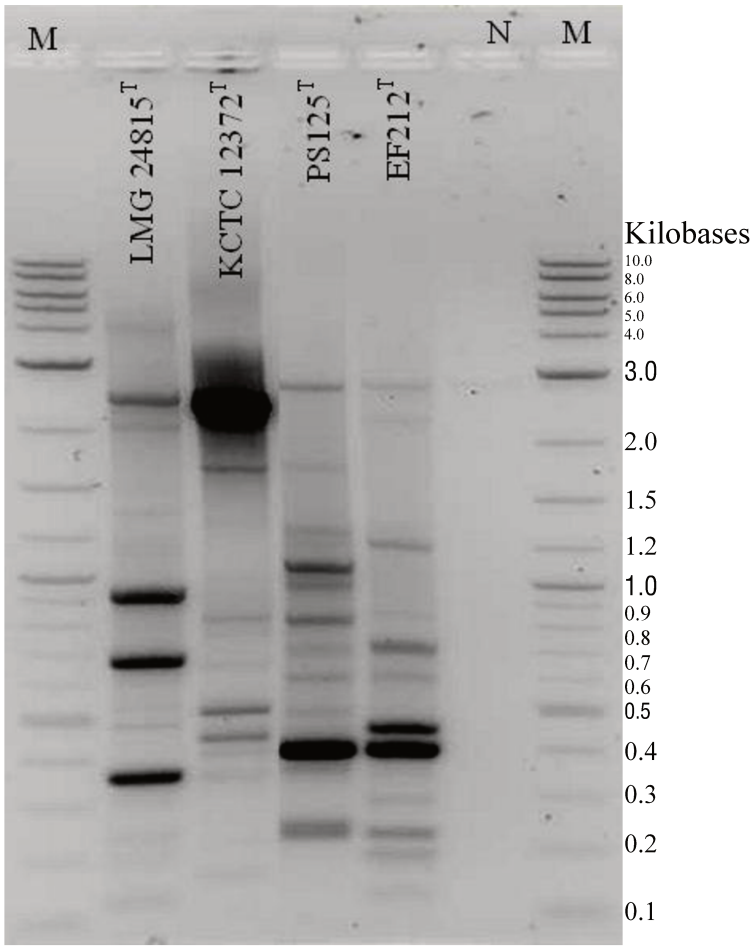


Figure 4.6 BOX-PCR fingerprinting of *Endozoicomonas* spp. BOX PCR performed in triplicate to ensure reproducibility of genomic DNA fingerprint.
Abbreviations: M = New England Biolabs 2-log ladder (0.1-10.0 kb); N = Negative control

Table 4.6 DNA-DNA hybridization results comparing genomic similarity of *Endozoicomonas* spp. in 2X saline-sodium citrate at 69 °C.

	E. euniceicola EF212^T	*E. gorgoniicola* PS125^T	*E. montiporae* LMG 24815^T
E. euniceicola EF212^T			
E. gorgoniicola PS125^T	28.4 (32.6)		
E. montiporae LMG 24815^T	33.5 (34.8)	21.9 (11.7)	
E. elysicola KCTC 12372^T	19.8 (28.2)	10.3 (4.8)	17.0 (15.2)[#]; 12.7 (14.5)[2]

Values in parentheses are results of duplicate measurements. Analysis carried out by DSMZ, Germany. [#]Data obtained in current study. [2]This is the result of reciprocal binding and labeling experiments and not a replicate DNA-DNA hybridization measurement, as was carried out in this study.

4.4 Formal Species Description

4.4.1 Emended Description of Endozoicomonas Genus

The description of the genus originally defined by Kurahashi and Yokota (2007),[1] with modifications by Nishijima *et al.* (2012),[3] should also be expanded to include the following characteristics. Colony color on MA may be beige or white. They can be strict aerobes or facultative anaerobes.[3] The major cellular fatty acids include summed feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$), summed feature 8 ($C_{18:1}\omega7c$), and $C_{16:0}$. All species also contain $C_{14:0}$ and 3-OH $C_{10:0}$, although in varying quantities. All other FAMEs are species dependent and not consistent across all species in presence or abundance. The G+C content should be further expanded to include 47.5 – 50.4 mol%. All species in the genus to date have been reported to utilize α-D-glucose and N-acetyl-D-glucosamine as a carbon source. In addition, Tween 40, Tween 80, and α-ketoglutaric acid are metabolized by all strains except NBRC 108893^T, but these carbon sources were not tested on this strain. As a general observation, all species to date have also been isolated from benthic, marine invertebrates.

4.4.2 Description of Endozoicomonas euniceicola sp. nov.

Endozoicomonas euniceicola (eu.ni.ce.i'cola. N. L. gen. fem. n. *Eunicea, Eunicea*, name of zoological genus; L. suff. *cola*, dweller; N. L. n. *euniceicola*, *Eunicea* dweller).

Cells are Gram-negative, motile rods, width 0.6-0.9 μm (average, 0.7 μm) and length 1.7-2.6 μm (average, 2.0 μm). Colonies on MA are white in color, circular, and convex. Colonies are 0.2-0.5 mm in diameter on MA after 72 h incubation at 25 ^oC. Growth occurs at 15-30 ^oC, at pH 7.0-8.0, with minimal growth < 1% and > 4% NaCl. Optimal growth occurs at 22-30 ^oC, at pH 8.0, with 2-3% NaCl. Cells are facultatively anaerobic. Positive for hydrolysis of Tween 20, oxidase, and catalase, and using API 20 NE, positive for the following enzymes: alkaline phosphatase, C4 esterase, C8 esterase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. A weak positive reaction is demonstrated for the hydrolysis of Tweens 40, 60, and 80, lipase activity, esculin hydrolysis (β-

glucosidase) (API ZYM), β-galactosidase (API ZYM), and trypsin (API 20 NE). Negative results are obtained for the following: DNase activity, starch hydrolysis, casein hydrolysis. In addition, using the API ZYM test strips, negative for nitrate reduction, indole production (tryptophan), D-glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis (protease); using the API 20 NE test strips, negative for lipase (C_{14}), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The following carbon substrates give strong positive results in the Biolog GN2 microplates: Tween 40, Tween 80, N-acetyl-D-glucosamine, α-D-glucose, α-keto glutaric acid, succinic acid, L-alaninamide, L-alanine, L-alanyl-glycine, L-proline, and L-threonine. The following carbon substrates give weak positive results in the Biolog GN2 microplates: dextrin, α-D-lactose, maltose, succinic acid mono-methyl-ester, acetic acid, bromosuccinic acid, L-asparagine, L-aspartic acid, glycyl-L-glutamic acid, inosine, uridine, and glycerol. The following carbon sources are not utilized in the Biolog GN2 microplates: α-cyclodextrin, glycogen, N-acetyl-D-galactosamine, adonitol, L-arabinose, D-arabitol, D-cellobiose, I-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, m-inositol, lactulose, D-mannitol, D-mannose, D-melibiose, β-methyl-D-glucoside, D-psicose, D-raffinose, L-rhamnose, D-sorbitol, sucrose, D-trehalose, turanose, xylitol, pyruvic acid methyl ester, cis-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto valeric acid, D,L-lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succininamic acid, glucuronamide, D-alanine, L-glutamic acid, glycyl-L-aspartic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-pyroglytamic acid, D-serine, L-serine, D,L-carnitine, γ-amino butyric acid, urocanic acid, thymidine, phenyethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, D,L-α-glycerol phosphate, α-D-glucose-1-phosphate, and D-glucose-6-phosphate. Cells are susceptible (>3 mm inhibition zone) to the antibiotics ampicillin,

chloramphenicol, gentamicin, kanamycin, nalidixic acid, rifampicin, streptomycin, novobiocin, and penicillin G. Cells are moderately susceptible (1-3 mm inhibition zone) to tetracycline. The principal fatty acids are summed feature 3 ($C_{16:1\omega6c}$ and/or $C_{16:1\omega7c}$) (44.0%), $C_{16:0}$ (17.1%), summed feature 8 ($C_{18:1\omega7c}$) (16.0%), and $C_{14:0}$ (13.8%). The major respiratory quinones under the evaluated conditions are ubiquinone Q9 (79%) and ubiquinone Q8 (22%). The DNA G+C content of EF212^T is 48.6 mol%. The type strain, EF212^T (=NCCB 100458^T=DSM 26535^T) was isolated from the octocoral, *Eunicea fusca*, collected off the coast of Florida at a depth of 12.5 m.

4.4.3 Description of Endozoicomonas gorgoniicola sp. nov.

Endozoicomonas gorgoniicola (gor.go.ni.i'cola. N. L. gen. fem. n. *Gorgonia*, name of zoological genus; L. suff. *cola*, dweller; N. L. n. *gorgoniicola*, *Gorgonia* dweller).

Cells are Gram-negative, motile rods, width 0.4-0.9 μm (average, 0.7 μm) and length 1.7-2.5 μm (average, 2.0 μm). Colonies on MA are creamy-white in color, circular, and convex. Colonies are 0.5-1.0 mm in diameter on MA after 72 h incubation at 25 ^oC. Growth occurs at 15-30 ^oC, at pH 7.0-9.0, with minimal growth < 1% and > 4% NaCl. Optimal growth occurs at 22-30 ^oC, at pH 8.0, with 2-3% NaCl. Cells are facultatively anaerobic. Positive for the hydrolysis of Tweens 20, 40, 60, and 80, oxidase, catalase, lipase, DNase, and using API 20 NE, positive for the following enzymes: alkaline phosphatase, C4 esterase, C8 esterase, leucine arylamidase, valine arylamidase, cystine arylamidase, acid phosphatase, and naphthol-AS-BI-phosphohydrolase. A weak positive reaction is demonstrated for esculin hydrolysis (β-glucosidase) (API ZYM), β-galactosidase (API ZYM), and trypsin (API 20 NE). Negative results are obtained for starch and casein hydrolysis. In addition, using the API ZYM test strips, negative for nitrate reduction, indole production (tryptophan), D-glucose fermentation, arginine dihydrolase, urease, gelatin hydrolysis (protease); using the API 20 NE test strips, negative for lipase (C14), α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase. The following carbon substrates give strong positive results in the Biolog GN2 microplates: α-cyclodextrin,

Tween 40, Tween 80, N-acetyl-D-glucosamine, α-D-glucose, α-D-lactose, lactulose, maltose, D-mannose, D-raffinose, α-keto glutaric acid, succinic acid, L-alaninamide, L-alanine, L-alanyl-glycine, L-asparagine, L-aspartic acid, L-glutamic acid, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-proline, γ-amino butyric acid, inosine, uridine, 2-aminoethanol, glycerol, D,L-α-glycerol phosphate, and α-D-glucose-1-phosphate. The following carbon substrates give weak positive results in the Biolog GN2 microplates: glycogen, N-acetyl-D-galactosamine, D-arabitol, I-erythritol, m-inositol, D-mannitol, D-melibiose, L-rhamnose, D-sorbitol, D-trehalose, turanose, xylitol, acetic acid, citric acid, D-gluconic acid, D-glucosaminic acid, β-hydroxybutyric acid, γ-hydroxybutyric acid, D,L-lactic acid, malonic acid, quinic acid, D-saccharic acid, bromosuccinic acid, succininamic acid, L-histidine, L-ornithine, putrescine, and 2,3-butanediol. The following carbon sources are not utilized in the Biolog GN2 microplates: dextrin, succinic acid mono-methyl-ester, L-threonine, adonitol, L-arabinose, D-cellobiose, D-fructose, L-fucose, D-galactose, gentiobiose, β-methyl-D-glucoside, D-psicose, sucrose, pyruvic acid methyl ester, cis-aconitic acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-glucuronic acid, α-hydroxybutyric acid, *p*-hydroxy phenylacetic acid, itaconic acid, α-keto butyric acid, α-keto valeric acid, propionic acid, sebacic acid, glucuronamide, D-alanine, hydroxy-L-proline, L-leucine, L-phenylalanine, L-pyroglytamic acid, D-serine, L-serine, D,L-carnitine, urocanic acid, thymidine, phenyethylamine, and D-glucose-6-phosphate. Cells are susceptible (>3 mm inhibition zone) to the antibiotics ampicillin, chloramphenicol, kanamycin, nalidixic acid, rifampicin, streptomycin, tetracycline, novobiocin, and penicillin G. Cells are moderately susceptible (1-3 mm inhibition zone) to gentamicin. The principal fatty acids are summed feature 3 ($C_{16:1\omega6c}$ and/or $C_{16:1\omega7c}$) (49.4%), summed feature 8 ($C_{18:1\omega7c}$) (19.5%), $C_{16:0}$ (17.1%), and $C_{14:0}$ (8.5%). The major respiratory quinones under the evaluated conditions are ubiquinone Q9 (72%) and ubiquinone Q8 (21%). The DNA G+C content of PS125^T is 47.5 mol%. The type strain, PS125^T (=NCCB 100438^T=CECT 8353^T) was isolated from the octocoral, *Plexaura* sp., collected off the coast of Bimini, The Bahamas at a depth of 17.0 m.

4.5 Discussion

In addition to being novel species, these bacteria potentially play important ecological roles in their octocoral hosts. A culture-independent study using 454-pyrosequencing[33] (Chapter 2) revealed that these bacteria were dominant members of the microbial community in their respective octocorals. Numerous culture-independent studies of marine invertebrates have shown similar dominance of *Endozoicomonas* spp.[33-49] (often incorrectly referred to as the uncharacterized genus *'Spongiobacter'* or simply referred to as *Gammaproteobacteria* clones). Previous studies have demonstrated that coral-associated *Endozoicomonas* strains likely contribute to the degradation of sulfur compounds (*e.g.* dimethylsulfoniopropionate [DMSP][50,51]) produced by endosymbiotic dinoflagellate microalgae[52] that reside in the invertebrate host. Furthermore, the genome sequence of a related member of the family *Hahellaceae*, *Hahella chejuensis*,[53] revealed that over 2% of the genome was devoted to genes associated with secondary metabolism (*e.g.* polyketide synthase and non-ribosomal peptide synthetase genes).[54] Thus, EF212^T and PS125^T may play a key role in the health of the octocoral holobiont though nutrient cycling and protection from invading pathogens. Results of genome sequencing of EF212^T and PS125^T (Chapter 5) will undoubtedly shed light on the role of these bacteria in their octocoral hosts.

4.6 References

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CHAPTER 5: GENOMIC INSIGHTS INTO THE METABOLISM AND FUNCTIONS OF *ENDOZOICOMONAS EUNICEICOLA* EF212^{T} AND *ENDOZOICOMONAS GORGONIICOLA* PS125^{T}, BACTERIA ASSOCIATED WITH THE OCTOCORALS, *EUNICEA FUSCA* AND *PLEXAURA* SP., RESPECTIVELY

5.1 Introduction

Endozoicomonas euniceicola EF212^T and *E. gorgoniicola* PS125^T were cultured from the octocorals *E. fusca* and *Plexaura* sp., respectively. Low DNA-DNA hybridization re-association values (4.8-34.8%) of EF212^T and PS125^T compared to with other characterized *Endozoicomonas* spp. (*E. elysicola*[1] and *E. montiporae*[2]), as well as differences observed in phenotypic, chemotaxonomic, and genotypic experiments compared to all other strains[1-3] verified that EF212^T and PS125^T were novel species (Chapter 4).[4]

EF212^T and PS125^T were shown to be abundant and ubiquitous associates in the octocoral microbiomes in a parallel culture-independent study (Chapter 2).[5] In addition, members of this bacterial genus have been reported to be prevalent in many other octocorals and corals from geographically diverse locations.[6-15] The abundance and ubiquity of *Endozoicomonas* spp. suggests that they may be essential to coral health, yet their function(s) and metabolic capabilities are completely unexplored to date. Thus, genome sequencing could provide insights into their roles within the holobiont and their metabolic capabilities.

To date, there is no formal genome description for the genus *Endozoicomonas*, however, *E. elysicola* DSM 22380^T and MKT110^T (NCBI Project ID: 11777) and *E. montiporae* CL-33^T (NCBI Project ID: 11499) genomes have been sequenced, and the annotation is in progress (http://www.ncbi.nlm.nih.gov/genome/?term=Endozoicomonas). The closest relative with a published, formal genome description is *Hahella chejuensis* KCTC 2396^T (family *Hahellaceae*, order *Oceanospirillales*, class *Gammaproteobacteria*).[16] This bacterium is in the same family as *Endozoicomonas*, but it is a free-living bacterium inhabiting marine sediments. Its 7.2 megabase pair (Mb) genome contains genes for a free-living, heterotrophic lifestyle and the algicidal agent, prodigiosin.[16]

It has been suggested that *Endozoicomonas* spp. may provide corals with essential nutrients,[6] cycle nutrients (*e.g.* sulfur) in the oligotrophic coral ecosystems,[10,17,18] and/or produce antimicrobial compounds.[12,13,19-22] In addition, we were particularly interested in determining if

the stable associate EF212^T, isolated from *E. fusca*, contained genes involved in fuscol[55] or related diterpene[56-58] biosynthesis. It is tempting to think that a bacterium with a stable association across geographical locations may be the biosynthetic source of the secondary metabolites isolated from the octocoral. Identification of the genes responsible for the production of fuscol and related diterpenes in the genome of a bacterium would pave the way for future investigations into their biosynthesis. Subsequent expression of the identified biosynthetic pathway into a heterologous host could lead to a sustainable source of these diterpenes, facilitating their development as possible therapeutic agents.

Due to a lack of time, the genome of PS125^T will be further explored in a study not encompassed in this PhD thesis. Thus, this chapter will focus solely on the EF212^T genome. The goals of this study were as follows. (1) Outline the overall primary metabolism and genomic composition of EF212^T. (2) Determine if EF212^T possesses complete secondary metabolite gene clusters, including those for diterpene biosynthesis. (3) Demonstrate genetic proof for previously proposed functions (dimethylsulfoniopropionate [DMSP] degradation) and bioactivity (antimicrobial). (4) Compare the genetic metabolism to the observed phenotypic metabolism previously described.[4] (5) Propose the potential functions of this ubiquitous, abundant, and ecologically-important group of coral-associated bacteria within the holobiont.

5.2 Materials and Methods

5.2.1 Sample Collection and Processing

A sample (~30 g) of *Eunicea fusca* was collected by SCUBA diving off the southeastern coast of Florida in June of 2009 and processed as previously described (Chapters 2 – 4) in order to culture associated microbes.[4] Strain EF212^T was purified as a single colony two weeks after initial plating from a 10^{-3} dilution of <51 μm particles plated onto Marine Agar (MA, BD Difco, VWR, Mississauga, ON). Bacterial cells were grown in nutrient broth with 3% (w/v) NaCl (Sigma-Aldrich, Oakville, ON) in two 1 l Fernbachs for three days. Cells were pelleted, and genomic DNA (gDNA) was extracted using the GenElute Bacterial Genomic DNA Kit (Sigma-

Aldrich, Oakville, ON). The gDNA was assessed for purity ($OD_{260/280}$=1.87) using a NanoDrop Spectrophotometer (ND-1000, NanoDrop Technologies, Inc., Wilmington, DE), and 50 µg of 50 ng μl^{-1} gDNA was sent to BGI China (Shenzhen, China) for genome sequencing, assembly, and preliminary bioinformatics analysis.

5.2.2 Genome Sequencing and Assembly by BGI China

Genome sequencing was carried out by BGI China using high-throughput Illumina sequencing technology using paired-end sequencing with 0.5, 2, and 6 kilobase pair (kb) inserts. Raw sequencing data was filtered to remove reads with ≥ 40% low quality base pairs, reads with ≥ 10% N base pairs, and adapter and duplication contamination. Sequences were then assembled using the software SOAP*de novo* v. 1.05 (http://soap.genomics.org.cn/soapdenovo.html). Read sequence overlap was determined using a de Bruijn graph. Contigs of similar sequences were created, and overlapping sequences were joined into 19 scaffolds based on the de Bruijn graph.

5.2.3 Bioinformatics Analysis by BGI China and In-House

The genomic sequences of the scaffolds were analyzed by BGI China for the following elements using the databases and software indicated in parentheses: (1) non-coding ribosomal nucleic acids (RNA) (rRNAmmer, tRNAscan, and Rfam software); (2) repetitive sequences (RepeatMasker software, using Repbase database and RepeatProteinMasker software, using the RepeatMasker library to predict transposons); (3) tandem repeats (Tandem Repeat Finder software); (4) genomic islands (Standalone BLAT v. 34 and SIGI-HMM software); (5) prophages (Standalone BLAT v. 34, Prophinder software, and ACLAME database); (6) CRISPRs (CRISPRFinder software); and (7) protein coding genes (protein sequences obtained from assembly using Glimmer3.0 software). The predicted functional genes were then aligned with five protein databases: Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of protein (COG), SwissProt, Translated of European Molecular Biology Laboratory Nucleotide Sequence Data Library (TrEMBL), and non-redundant protein sequences (NR) in the GenBank database to obtain corresponding functional annotation.

Further processing of the functional genes and identification of homologous gene clusters of interest was carried out in-house using BLASTp (National Center for Biotechnology Information [NCBI], Bethesda, MD) and European Nucleotide Archive (ENA, European Bioinformatics Institute, Cambridge, UK) database searches. In addition, secondary metabolite gene clusters were detected using antiSMASH.[23] The draft genome of EF212^T will be available under the NCBI Project ID: 15585.

5.3 Results

5.3.1 General Features of the EF212^T Genome

The genome of *E. euniceicola* EF212^T was assembled into 19 scaffolds, with an estimated genome size of 6,348,396 base pairs (bp). Among the 5,788 predicted genes, 2,756 (47.6 %) were assigned a putative function, 731 plus an additional 45 (13.4 % together) had hypothetical or conserved hypothetical functions, respectively, and 2,256 (39.0 %) had unknown or not available (N/A) functions according to the GenBank 'nr' database. Table 5.1 lists the general genome features of EF212^T.

Overall, based on the KEGG pathway classification of EF212^T (Figure 5.1), this bacterium has an abundance of environmental information processing and membrane transport genes (Figure 5.1, light orange box), as well as many genes involved in amino acid (Figure 5.1, blue box) and carbohydrate metabolism (Figure 5.1, dark purple box). It also has a number of genes involved in genetic information processing (Figure 5.1, replication, repair, and translation; green bars), and energy metabolism (Figure 5.1, purple bar). In addition, EF212^T has genes involved in the biosynthesis of polyketides and terpenoids (Figure 5.1, red bar), although these genes are not as abundant as the primary metabolism genes.

Below is the COG function classification graph (Figure 5.2). This graph shows that there are an abundance of genes with only a general function predicted (Figure 5.2, blue box), genes involved in replication, recombination, and repair (Figure 5.2, aqua box), as well as many genes for amino acid metabolism and transport (Figure 5.2, yellow box).

Table 5.1. General features of the *Endozoicomonas euniceicola* EF212^T genome.

No. of scaffolds	19
Length of genome assembly (bp)	6,348,396
G+C content of genome sequence (%)	48.1
No. of predicted protein-coding genes	5,788
Length of protein-coding gene region (bp)	5,555,043
Percentage protein-coding of total genome (%)	87.5
Average protein-coding gene length (bp)	959
GC content of protein-coding genes (%)	48.7
CDS Summary	
Assigned function	2,756
Conserved hypothetical (function predicted)	45
Hypothetical (unclassified/poorly characterized)	731
Unknown function (N/A)	2,256
Length of intergenic region (bp)	793,353
Percentage of intergenic region of total genome (%)	12.5
GC Content of intergenic region (%)	44.1

Abbreviations: No. = number; CDS = coding DNA sequence; N/A = not available; bp = basepair

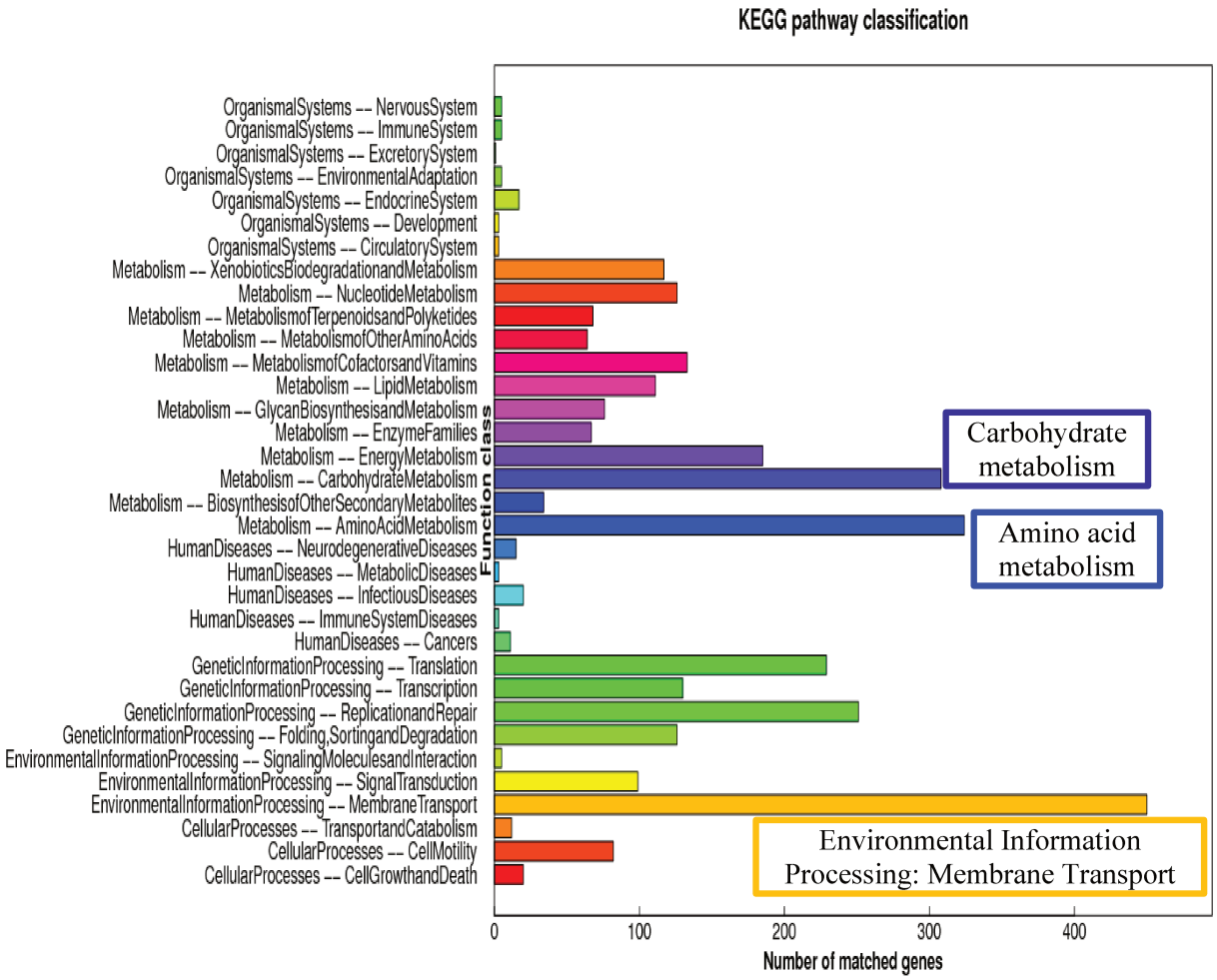

Figure 5.1 Kyoto encyclopedia of genes and genomes (KEGG) pathway classifications of EF212^{T} annotated genes. The most abundant functional classes of genes are in the colored boxes.

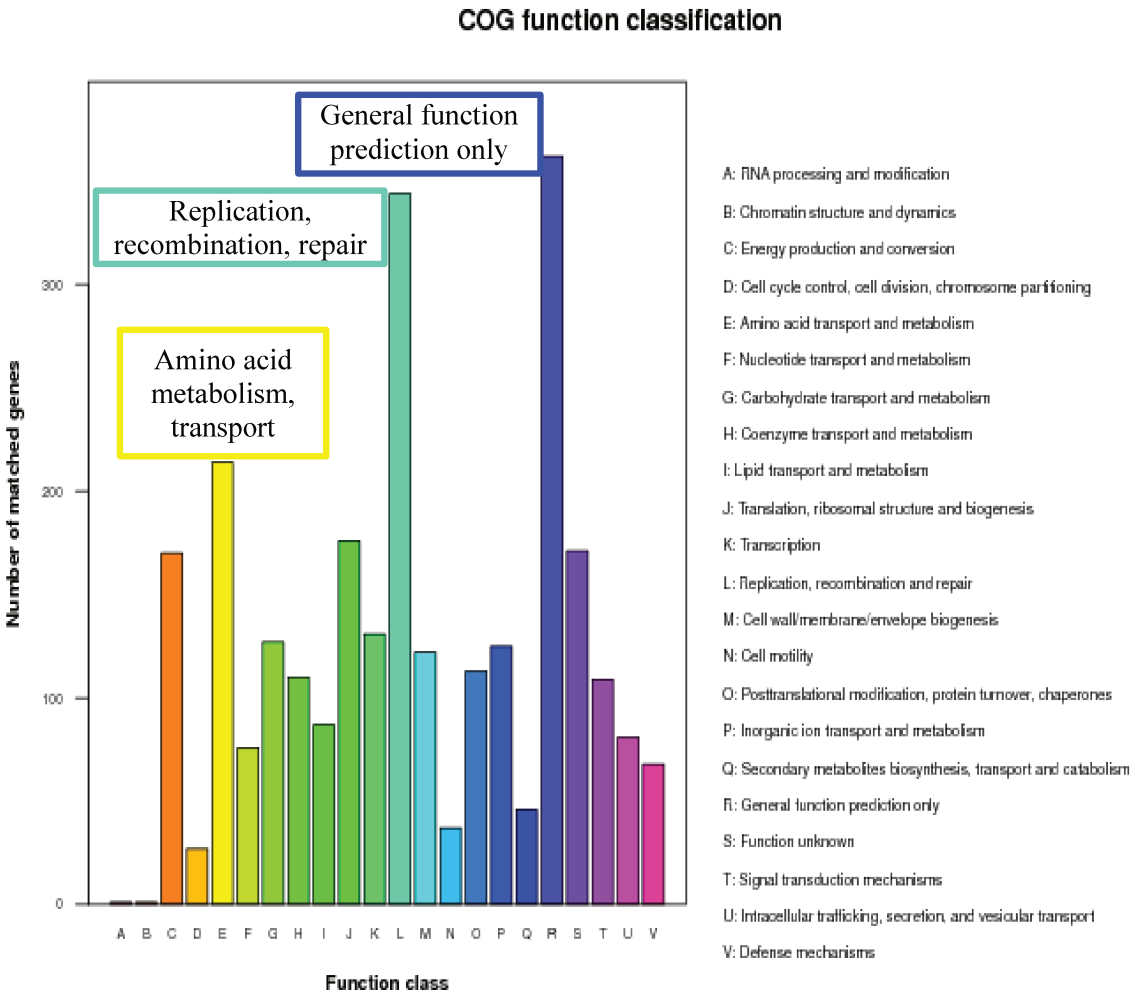

Figure 5.2 Clusters of orthologous groups of protein (COG) function classification of EF212^T genes. The most abundant functional classes of genes are in the colored boxes.

5.3.2 EF212^T Genes Involved in Primary Metabolism

A closer look at EF212^T's genes annotated using the five protein databases (KEGG, COG, SwissProt, TrEMBL, and NR) suggests that there are many genes involved in central carbon metabolism (TCA cycle, glycolysis, oxidative phosphorylation, and pentose phosphate pathway). In addition, EF212^T has genes involved in anaerobic metabolism (*e.g.* G-3-P dehydrogenase, sulfite reductase, dimethyl sulfoxide reductase, and coproporphyrinogen III oxidase). EF212^T has genes for the biosynthesis of nucleotides, for the metabolism and/or biosynthesis of the 21 standard amino acids, and for fatty acid biosynthesis and metabolism. This bacterium has the ability to metabolize many carbohydrates, nitrogen (reduction and fixation), sulfur (reduction and fixation), and methane. Extracellular hydrolytic enzymes involved in the degradation of organic (proteases, lipases, nucleases, chitinases) and inorganic (carbon monoxide dehydrogenase, sulfur reductase, sulfite oxidase) substrates are present. Genes for N-glycan, lipopolysaccharide, and peptidoglycan biosynthesis, as well as glycerolipid, glycerophospholipid, and inositol phosphate metabolism are also found in EF212^T. This bacterium has genes for the metabolism and biosynthesis of a number of B vitamins, including thiamine (B_1), riboflavin (B_2), nicotinate and nicotinamide (B_3), pantothenate (B_5) and CoA biosynthesis, pyridoxine (B_6), biotin (B_7), and folate (B_9).

Finally, EF212^T has genes involved in the demethylation and cleavage of the dinoflagellate photosynthetic byproduct, DMSP. DMSP is transported into the cell via the DddT protein (Figure 5.3) and can undergo one of two fates for degradation: demethylation (Figure 5.4) or cleavage (Figure 5.5).[24]

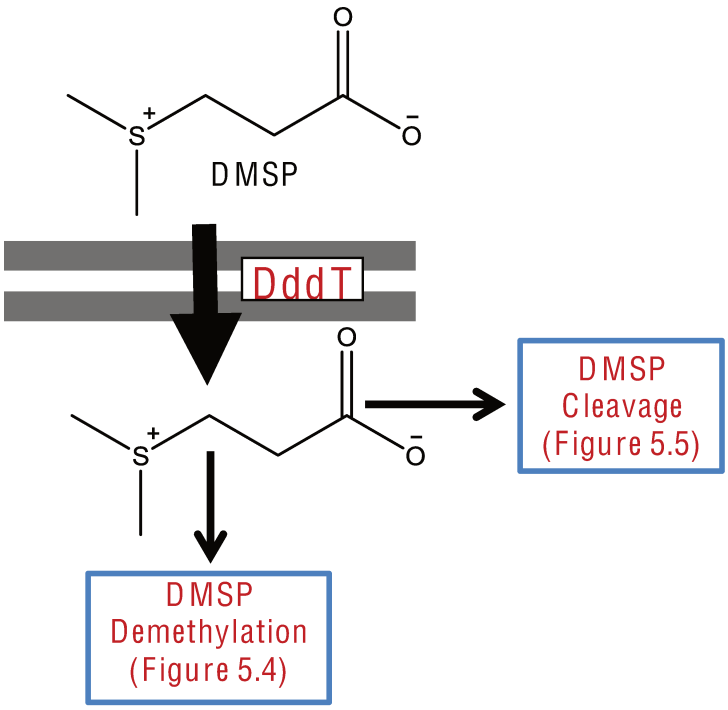

Figure 5.3 Dimethylsulfoniopropionate (DMSP) transport into the bacterial cell. DMSP is transported through the EF212^{T} membrane using the protein transporter DddT. After being actively transported across the membrane, it can undergo a demethylation or cleavage pathway. The DddT protein and the demethylation and cleavage pathways are present in EF212^{T}. Image adapted from Todd *et al.*, 2010.[59]

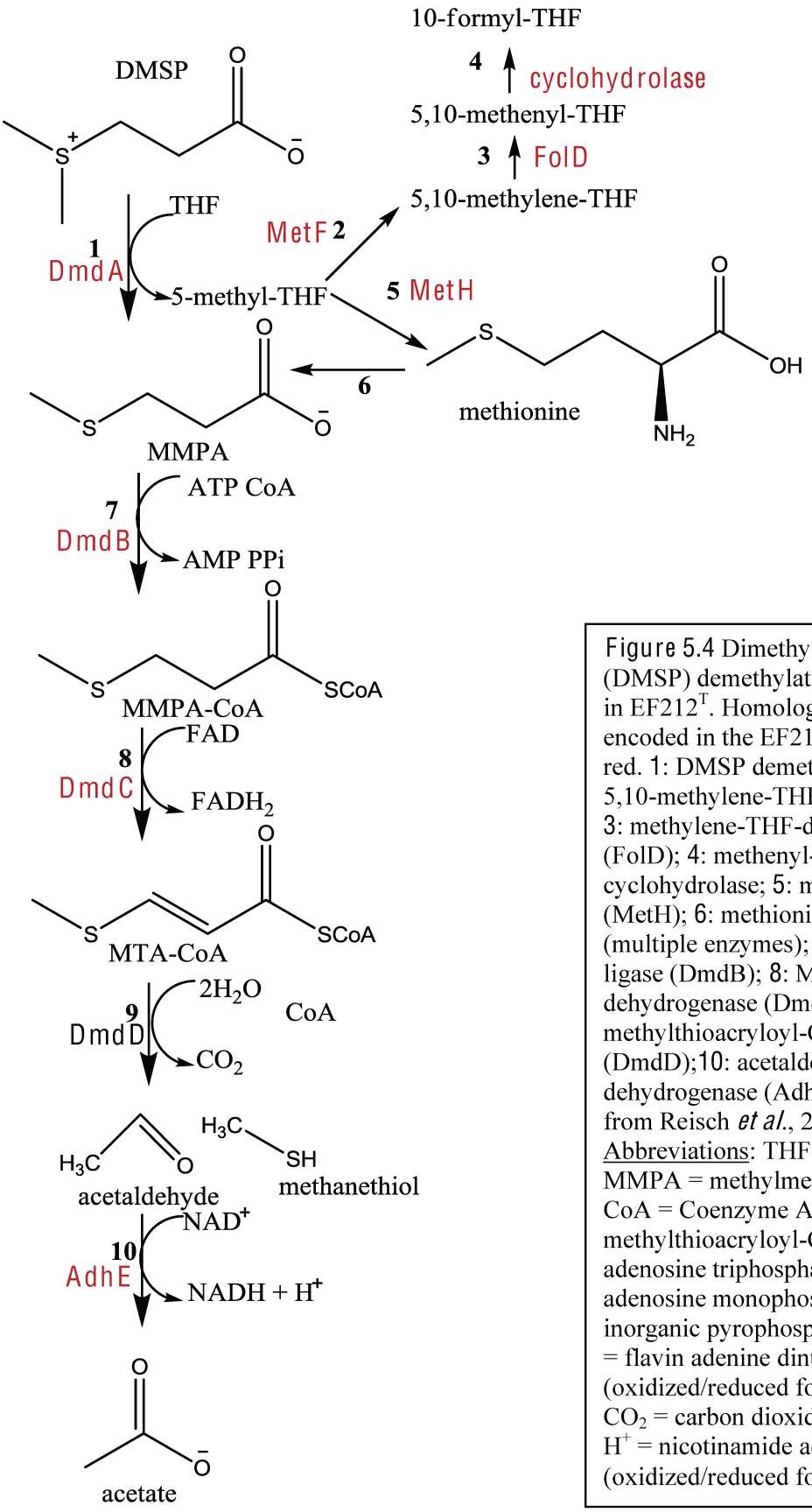

Figure 5.4 Dimethylsulfoniopropionate (DMSP) demethylation pathway present in EF212[T]. Homologous proteins encoded in the EF212[T] genome are in red. 1: DMSP demethylase (DmdA); 2: 5,10-methylene-THF-reductase (MetF); 3: methylene-THF-dehydrogenase (FolD); 4: methenyl-THF-cyclohydrolase; 5: methionine synthase (MetH); 6: methionine salvage pathway (multiple enzymes); 7: MMPA-CoA ligase (DmdB); 8: MMPA-CoA dehydrogenase (DmdC); 9: methylthioacryloyl-CoA hydratase (DmdD);10: acetaldehyde dehydrogenase (AdhE). Image adapted from Reisch *et al.*, 2011.[24]
Abbreviations: THF = tetrahydrofolate; MMPA = methylmercaptopropionate; CoA = Coenzyme A; MTA-CoA = methylthioacryloyl-CoA; ATP = adenosine triphosphate; AMP = adenosine monophosphate; PPi = inorganic pyrophosphate; FAD/$FADH_2$ = flavin adenine dinucleotide (oxidized/reduced forms); H_2O = water; CO_2 = carbon dioxide; NAD^+/NADH + H^+ = nicotinamide adenine dinucleotide (oxidized/reduced forms)

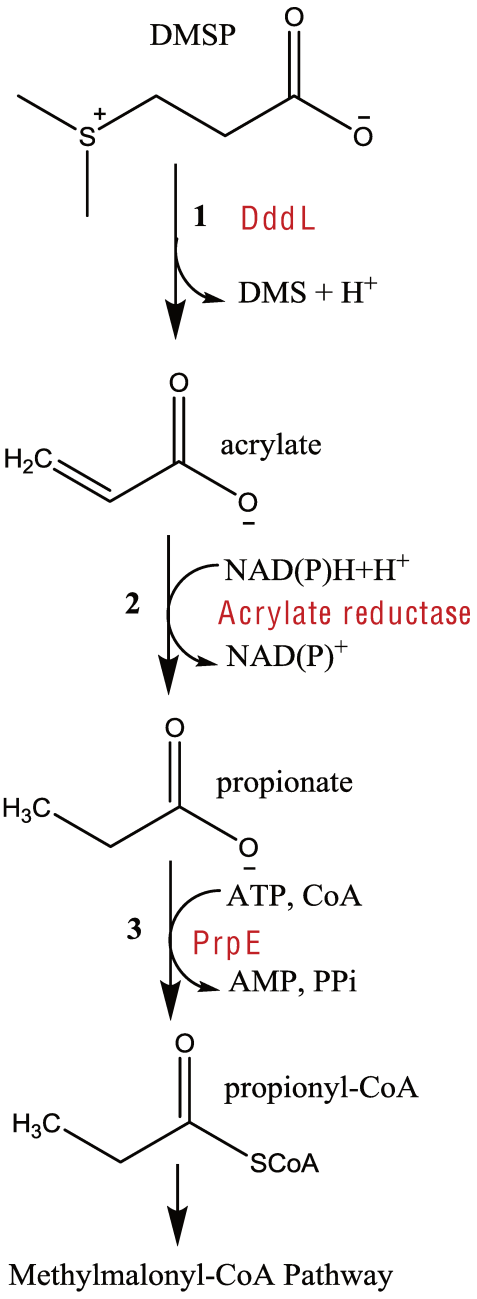

Figure 5.5 Dimethylsulfoniopropionate (DMSP) cleavage pathway present in EF212^{T}. Homologous proteins encoded in the EF212^{T} genome are in red. 1: DMSP lyase (DddL); 2: acrylate reductase; 3: propionate-CoA ligase (PrpE). Image adapted from Reisch *et al.*, 2011.[24] Abbreviations: DMS = dimethyl sulfide; H^{+} = proton; NAD(P)H/NAD(P)$^{+}$ = nicotinamide adenine dinucleotide phosphate (oxidized/reduced forms); ATP = adenosine triphosphate; AMP = adenosine monophosphate; PPi = inorganic pyrophosphate; CoA = Coenzyme A

5.3.3 EF212^T Genes Involved in Secondary Metabolism

All of the genes involved in the terpene deoxyxylulose pathway were found, but the presence of ubiquinone and menaquinone biosynthetic genes suggests that this terpene precursor biosynthetic pathway is likely involved in the biosynthesis of these isoprenoid quinones involved in aerobic and anaerobic respiration, respectively. No terpene synthases for fuscol biosynthesis were detected.

EF212^T has multiple independent genes where the same function is redundantly encoded (*e.g.* Type II and III secretion systems). These are likely encoded by horizontally transferred genes (HTGs) from other bacteria and species.[16] There are also an abundance of mobile elements (*e.g.* transposases, Rhs elements, bacteriophages, and group II introns) associated with HTGs on genomic islands (GIs). These GIs contain complete or incomplete secondary metabolite gene clusters encoding for one of the following: (a) antimicrobial biosynthesis (complete pathway for bacteriocin biosynthesis and export; almost complete pathway for streptomycin biosynthesis; partial pathways for tetracycline, penicillin, cephalosporin, novobiocin, ansamycin, and vancomycin biosynthesis); (b) antimicrobial resistance (β-lactam, bicyclomycin/chloramphenicol, icyclomycin/chloramphenicol, glyoxalase/bleomycin, tellurite, and multiple antibiotic resistance C [marC] protein) (c) toxin/antitoxin systems (antitoxin [MazE] protein, hemagglutinin/hemolysin); (d) capsular exopolysacchrides biosynthesis and export (biofilms/virulence factors, peptidoglycan biosynthesis protein [mviN]); (e) complete siderophore biosynthesis pathway (iron utilization); (f) motility; (g) secretion systems (type I, II, III, IV, V, and VI) and transporters (adenosine triphosphate [ATP]-binding cassette [ABC] and tripartite ATP-independent periplasmic [TRAP], and multidrug efflux pumps); (h) degradation of organic compounds (polycylic aromatic hydrocarbons, naphthalene, carbazole, ethylbenzene, caprolactam, dioxin, xylene, toluene, chloroalkane/-alkene, aminobenzoate, nitrotoluene, styrene, dichlorodiphenyltrichloroethane, chlorocyclohexane and chlorobenzene benzoate, bisphenol); (i) metabolism and biodegradation of xenobiotics and drug metabolism (cytochrome p450s,

azathioprine and 6-mercaptopurine degradation, fluorouracil degradation); (j) miscellaneous (plasmid maintenance system and V-defense mechanisms).

5.3.4 EF212^T Genes Involved in Environmental Interaction

As observed from the KEGG graph, there are an abundance of genes involved in environmental sensing, regulation, and membrane transport. There are a number of environmental sensor and response regulatory genes for a number of different functions (*e.g.* limitation of nitrogen and phosphate, chemotaxis, osmotic regulation, citrate fermentation, cell-cycle regulation, pili twitching motility, C4-dicarboxylate transport, anaerobic C4-dicarboxylate transport, and carbon storage). Also present are genes encoding proteins involved in transcriptional regulation (LysR, AraC, TetR, and MerR), as well as σ factors (σ70 for housekeeping, σ32 for heat shock, σ54 for nitrogen limitation, and σ28 for flagellar regulation), which initiate transcription according to environmental conditions. There are a number of membrane transport systems (ABC transport system, two-component system, phosphotransferase systems, Na^+ transporters, and Na^+ translocating respiratory NADH:ubiquinone oxidoreducatase transporters) for the transfer of sugars, peptides, amino acids, nitrogen, ammonia, phosphate, iron, zinc, copper, sulfur, manganese, sodium, molybdate, nickel, methane, B vitamins, and many more nutrients.

EF212^T also has genes involved in quorum sensing (LuxR family response regulators; AI-2 TqsA), chemotaxis and motility (Rsb, MalE, CheY, Fil, Mot, Pil), sporulation/biofilm formation (Spo0), and a phosphatidylinositol signaling system.

5.4 Discussion

The genetic repertoire of EF212^T suggests that it is a heterotrophic, marine bacterium that is host-interactive and likely symbiotic with the *E. fusca* holobiont (including octocoral, zooxanthellae, and other associated microbes). The abundance of environmental sensing and transport genes, as well as the functionally diverse metabolism of EF212^T, suggest that it may provide the *E. fusca* holobiont with nutrients that the octocoral itself cannot assimilate or obtain from the oligotrophic ocean environment. In addition, there is evidence that EF212^T is a recent, facultative symbiont in the early stages of association with *E. fusca*.

5.4.1 Genomic Evidence of Nutritional Provision by EF212^T

Over the past decade, many symbiotic relationships between bacteria and invertebrates have been studied via genomics. Most of these symbioses involve *Gammaproteobacteria* that live in an obligate association with a specific invertebrate.[26] These bacterial endosymbionts complement the limited metabolic capabilities of the invertebrates through the provision and recycling of nutrients. This may include nitrogen cycling (fixation, assimilation, recycling, and storage), sulfur cycling, methanogenesis, chemolithoautotrophy, and essential-nutrient anabolism, including the biosynthesis of the essential amino acids, vitamins and cofactors, and fatty acids that are deficient in the host diet and metabolic repertoire.[17,18,26] In particular, invertebrate heterotrophic metabolism is relatively limited in its biosynthetic capabilities of some amino acids, vitamins, and fatty acids, so they must be obtained from an external source.[26]

It is thus hypothesized that EF212^T supplies the octocoral with nutrients (*e.g.* nitrogen, sulfur, amino acids, B vitamins, and fatty acids) that the octocoral itself cannot assimilate or obtain from the oligotrophic environment, but are required for survival. EF212^T is also likely involved in host-waste nutrient recycling. EF212^T uses both chemoorganoheterotrophic (*i.e.* organic energy sources) and chemolithoheterotrophic (*i.e.* using inorganic nutrients when organic ones are scarce) metabolism to break down environmental nutrients, and it also has the ability to biosynthesize all of the essential amino acids and many B vitamins. It can then provide all of

these nutrients and/or other metabolic byproducts to the octocoral. The previous identification of *Gammaproteobacteria* aggregates (likely *Endozoicomonas* relatives) within the gastrodermal layer of the digestive cavity of several reef-building corals supports the hypothesis that these bacteria play a key role in the octocoral's nutrient provision and recycling.[27]

Endozoicomonas spp. associated with zooxanthellate coral have also been shown to possess the ability to metabolize the organosulfur compound DMSP, suggesting a role in sulfur-cycling within these corals.[6,17,18] DMSP is produced in large quantities by coral endosymbiotic dinoflagellates, *Symbiodinium* spp.,[28] and is found in significant concentrations in zooxanthellate coral.[29,30] Thus, the presence of genes encoding both the demethylation and cleavage pathway of DMSP in EF212^T is not surprising. EF212^T is therefore able to provide the coral with additional nutrients from the degradation of DMSP, as well as decrease the concentration of this dinoflagellate waste product within the holobiont.

The abundance of high-affinity ABC and TRAP transporters provides additional evidence of nutritional provision to the holobiont. These systems are used to actively transport a variety of smaller inorganic compounds and larger organic substrates (*e.g.* amino acids, peptides, and sugars) against a large concentration gradient while using ATP energy or an ion gradient.[31] ABC and TRAP transporters are common in bacteria from oligotrophic environments, such as the ocean, since these transporters have extremely high affinities for substrates at extremely low concentrations.[31] The high abundances of these transporters in EF212^T allows it to quickly respond to and take up many different substrates that may be present at low concentrations in the marine environment. It likely uses the substrates as an energy source, transports them directly to the octocoral, or uses them as a source of building blocks for downstream metabolic products.

5.4.2 Genomic Evidence for Secondary Metabolism

No genes for isoprenoid synthases for fuscol biosynthesis were detected in the ~6.3 Mb draft genome of EF212^T. However, diterpene biosynthetic pathways are often 10-15 kb in size, so the lack of closure of the genome may have caused the genes to be eliminated from the dataset, or

incomplete genes may have been unidentifiable using typical database searches. However, it is likely that fuscol is biosynthesized by (an)other member(s) of the holobiont, such as the octocoral *E. fusca*, the *Symbiodinium* symbiont, or another stable microbial symbiont (*e.g.* the *Mycoplasma*-relative[5] discussed in Chapter 2), instead of EF212^T.

Even though diterpene biosynthetic genes were not present, EF212^T possessed complete pathways for bacteriocin, most closely related to aerobactin, biosynthesis and transport (Figure 5.6: Scaffold 1, gene position 1256181 – 1267095 and annotated proteins B212GL001256-63) and siderophore biosynthesis and transport (Figure 5.7: Scaffold 1, gene position 1503594 – 1518324 and annotated proteins B212GL001477-84).

Bacteriocins are ribosomally synthesized antibiotic peptides, commonly found in marine bacteria associated with invertebrates.[32] They are toxic to closely-related bacteria, and therefore may provide a probiotic effect by preventing the invasion of unwanted bacteria in an established microbial community. Bacteriocins may also act as communication or quorum sensing molecules in dense bacterial communities.[32] Previously observed antimicrobial activity from *Endozoicomonas* spp.[20-22] may be caused by the presence of bacteriocins.

Iron is limited in the marine environment, but it is essential for many biological processes, including the growth of bacteria. Thus, marine bacteria require a system to extract iron from the surrounding environment and sequester it for use. Many marine bacteria accomplish this by using siderophores,[33] low molecular weight iron (Fe)-binding chelators that can remove ferric iron (Fe [III]), heme, or hemoglobin from iron-binding proteins, [34] such as transferrin and lactoferrin, and ferritin. In Gram-negative bacteria, Fe (III), heme, or hemoglobin is chelated with a siderophore and transported into cells via a TonB-dependent uptake system, made up of an outer membrane receptor protein and an ABC transporter.[34] This TonB-dependent system is present in EF212^T, and likely assists EF212^T in the uptake of limited iron from the marine environment.

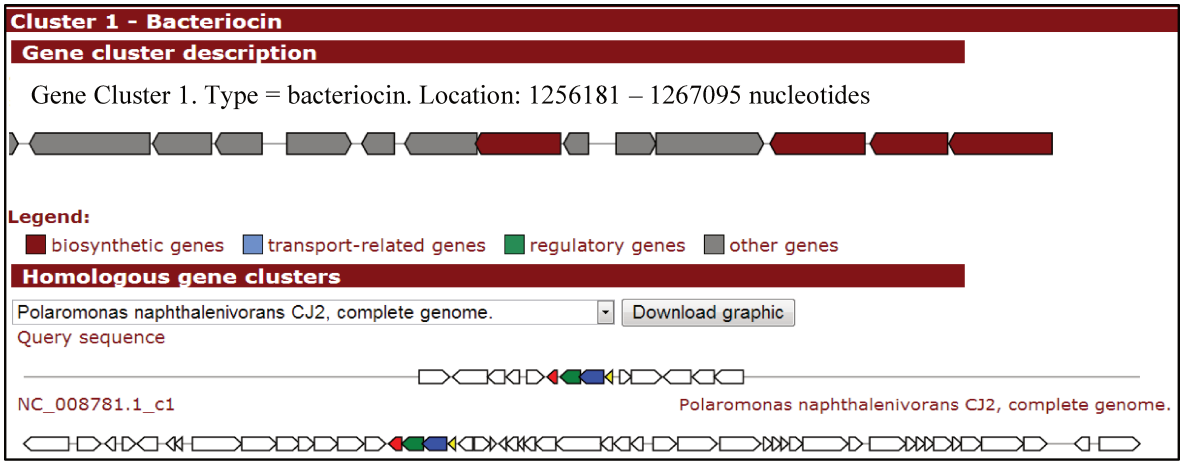

Figure 5.6 Antibiotics and secondary metabolite analysis shell (AntiSMASH) prediction of the bacteriocin gene cluster of EF212^{T}. The closest related homologous gene cluster is from *Polaromonas naphthalenivorans* CJ2. Image adapted from AntiSMASH.[23]

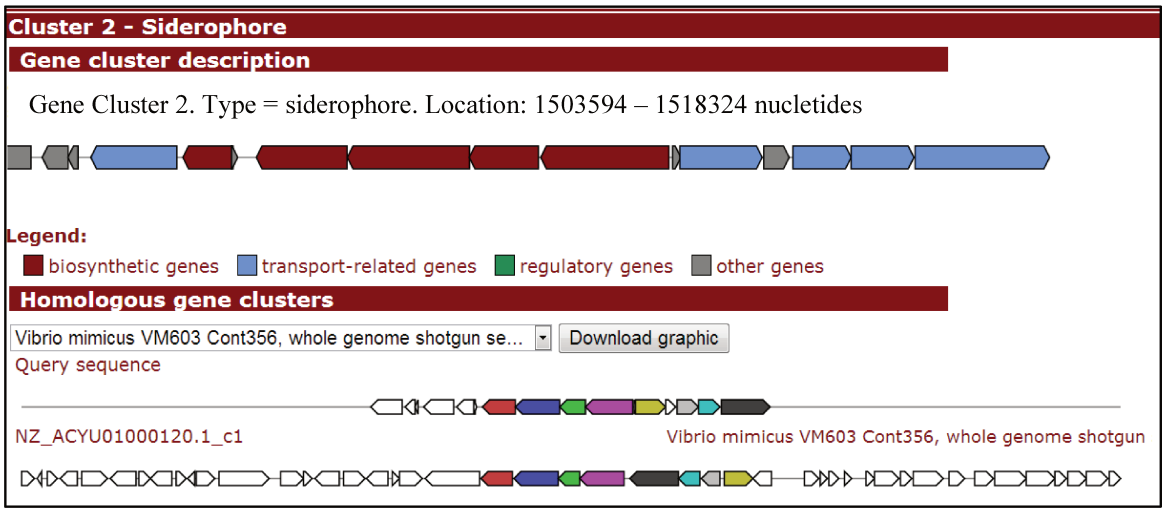

Figure 5.7 Antibiotics and secondary metabolite analysis shell (AntiSMASH) prediction of the siderophore gene cluster of EF212^{T}. The closest related homologous gene cluster is from *Vibrio mimicus* VM603. Image adapted from AntiSMASH.[23]

All other genes that were annotated as biosynthetic gene clusters for secondary metabolism contain incomplete pathways. These gene clusters are on GIs and were likely obtained through the incorporation of mobile genetic elements (*i.e.* plasmids, transposons, and bacteriophages) and transferred from other bacteria or microbes.

5.4.3 Similarities between Phenotypic and Genotypic Metabolism of EF212^T

The genome of EF212^T contains many genes that are observed though phenotypic expression. For example, it contains many genes for the metabolism of different carbon sources (*e.g.* α-D-glucose, N-acetyl-D-glucosamine, and succinic acid, maltose) and amino acids (*e.g.* L-alanine, aspartate, proline, threonine) that were shown to be metabolized in phenotypic experiments.[4] As well, there are genes for motility and chemotaxis, NaCl utilization, and anaerobic metabolism (Table 5.2) that were also confirmed by phenotypic experiments.[4]

Having genetic proof of these phenotypic characteristics will facilitate isolation and manipulation of related strains in future experiments. For example, knowledge of the preferred carbon sources can be used to design media to specifically isolate *Endozoicomonas* spp.

In addition to these primary metabolic characteristics, it was observed that EF212^T was moderately susceptible to the antibiotic, tetracycline (30 μg), with an inhibition zone of only 2 mm.[4] There are several mechanisms of bacterial resistance to antibiotics, such as inactivation of inhibitors, prevention of antibiotic influx into the cell, and the efflux of the antibiotic from the cell.[35] Multidrug transporters are one type of efflux pump that have been reported to remove a variety of toxins.[36] EF212^T contains a number of multidrug resistant proteins and transporters, and thus, one of these genes could provide moderate resistance to tetracycline through efflux of the antibiotic.

Table 5.2 Examples of genetic proof for previously observed phenotypic characteristics.

Observed Phenotypic Trait	Genes
Flagellum presence/motility	fliN, fliA, fliO, fliC, flgL, flgI, flgK, flrA, cheA, cheB, cheR, motB
NaCl requirement	Sodium and chloride channels, symporters, exchangers, pumps
Facultative anaerobic metabolism	dcuA, dcuB, dcuC, dmsA, dmsA, dmsB, glpA, glpB, glpC, nrdD, nrdG, asrA, asrB, asrC,

Tetracycline resistance has been previously observed in many Gram-negative, *Gammaproteobacteria* of marine origin,[37] including bacteria in the Order *Oceanospirillales* (*e.g. Saccharospirillum aestuarii*,[38] *Neptunomonas japonica*,[39] and *Kangiella koreensis*[40]). This may be caused by proximity to fish farms where antibiotic treatment is common and can lead to increased concentrations of tetracycline in the surrounding marine environment resulting in a positive selection for antibiotic-resistant bacteria in that area.[37] In addition to aquaculture, run-off pollution from sewage treatment plants and recreational beach areas may also contribute to the tetracycline antibiotic resistance of marine bacteria in a particular area.[37] EF212^T was isolated from an *E. fusca* (EF-FL2-C) off the southeast coast near Fort Lauderdale, FL (Figure 5.8, blue marker) where a number of fish farms (Figure 5.8, orange markers) and public beaches (Figure 5.8, red makers) are located, so both aquaculture or beach run-off may have contributed to and selected for moderate tetracycline resistance in EF212^T.

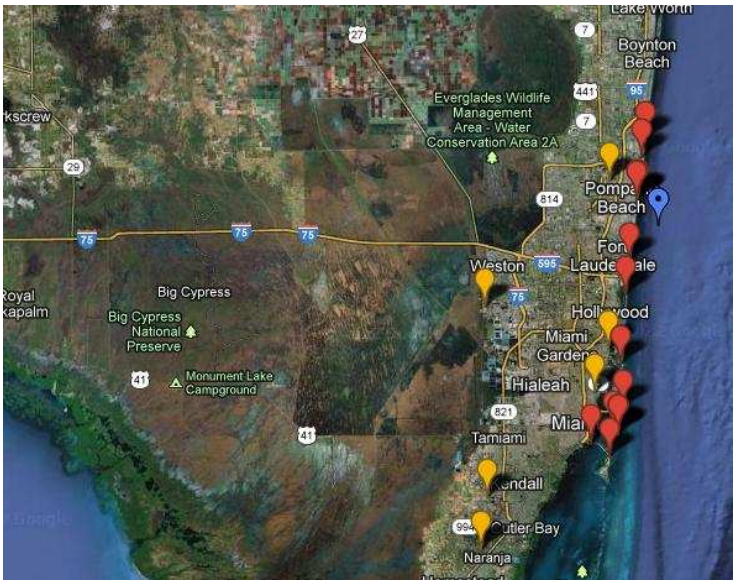

Figure 5.8 Map of fish farm locations (orange makers) and public beaches (red makers) near the collection site of octocoral EF-FL2-C (from which EF212^T was isolated) in Florida (blue maker). The proximity of EF212^T to these coastal sources of pollution may have caused the selection for moderate tetracycline resistance. Image obtained from Google Maps©.

5.4.4 Genomic Evidence for an Early Stage of Symbiosis

It is hypothesized that EF212^T is in an early, facultative stage of symbiosis (Figure 5.9) with *E. fusca*. This primary stage is supported by four main characteristics:

(1) EF212^T has a relatively large genome size compared to many ancient, obligate symbionts.[26] However, its genome is reduced compared to its free-living relative, *H. chejuensis*, which has a genome size of 7.2 Mb,[16] and is thus likely in the early stage of genome reduction. During the facultative stage of symbiosis, genome shrinkage gradually eliminates genes scattered throughout the genome, and inactivation of protein-coding genes (pseudogenization) by mutations occurs until the gene is completely eliminated in advanced, obligate stages of symbiosis.[26,41] Overtime, it is hypothesized that the EF212^T genome will decrease in size and only retain the pathways that are necessary to biosynthesize the essential nutrients that the octocoral holobiont requires and is unable to provide for itself.[26]

(2) The early stage of symbiosis is also supported by discrepancies in the protein coding regions. There is a slight difference in the GC content of the coding (~48.7%) and non-coding (~44.1%) regions.[42] This decrease in GC content in non-coding regions is often observed in established endosymbionts.[42] In addition, many of the hypothetical open reading frames (ORFs) are shorter than the majority of the coding sequences.[42] These hypothetical ORFs may be the non-functional pseudogenes often seen in genomes in the early stages of reduction.[26,43,44]

(3) There is an abundance of HTG on mobile genetic elements. The EF212^T genome has a predicted 379 mobile genetic element genes encoding for transposons, transposases, plasmids, phages, prophages, bacteriophages, which constitute 6.5 % of its genome. Mobile elements make up ~25% of the genomes of *Sitophilus oryzae* and *S. zeamais*, primary endosymbionts of the rice and maize weevils, respectively, and both have recently established obligate associations with their hosts.[26] Newly established, facultative endosymbionts contain four to five times more mobile DNA than obligate endosymbionts due to an increase in the rearrangement of elements at the start of the symbiosis.[26,45]All analyzed endosymbiotic bacteria that are involved in older

associations have genomes free of mobile elements, so the increase in mobile elements eventually becomes detrimental, and they are removed as part of the genome degradation process.[26]

(4) There are changes within the EF212^{T} genome, including rearrangements, gene duplication, recombination, functional divergence, and non-functionalization. These alterations have caused widespread genomic rearrangement, which will lead to successive gene inactivation (pseudogenization),[45-47] and these genes will eventually be lost or transferred to the host in late stages of symbiosis (Figure 5.9).[45]

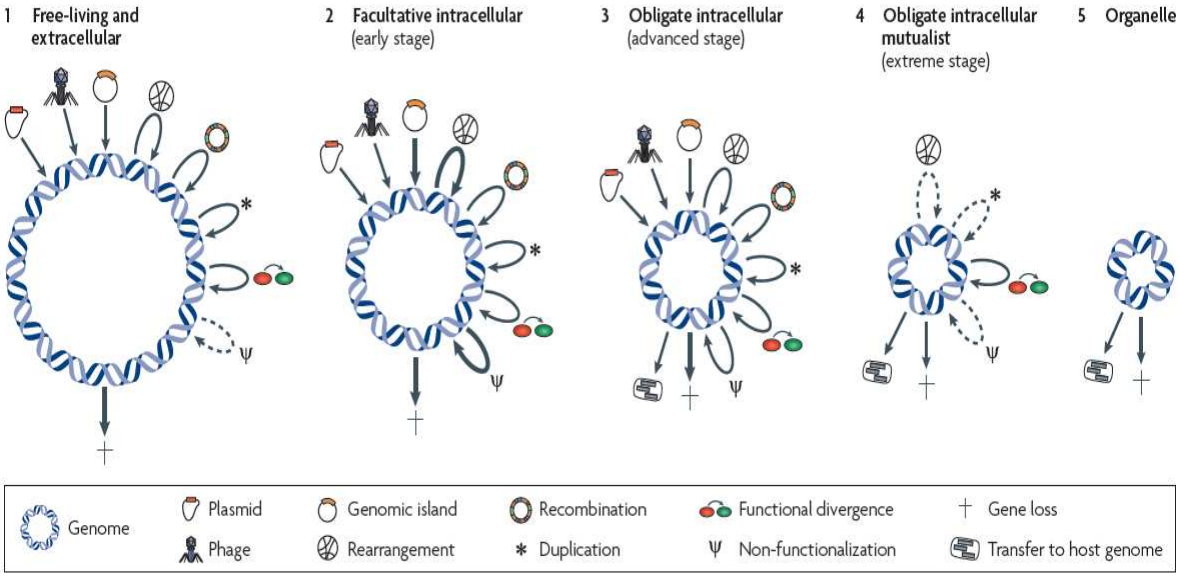

Figure 5.9 Stages of symbiotic bacterial adaptation. It is hypothesized that EF212^{T} is currently in the early, facultative stage (2) of symbiosis. Arrows pointing towards the genome indicate acquisition of HTG, those looping back towards the genome indicate genomic changes, and those pointing away from the genome indicate gene loss or transfer to the host genome. Image adapted from Toft & Anderson, 2010.[45]

5.4.5 Genomic Evidence for EF212^T Interaction with Host Cells

Despite the fact that EF212^T is in an early, facultative stage of symbiosis, it does contain many genes indicative of an established symbiotic or pathogenic bacterium. Both symbiotic and pathogenic bacteria often contain various secretion systems that allow them to attach to, invade, interact with, and maintain their presence within host cells.[26,47] They are often located within GI and near to mobile genetic elements.[47] EF212^T encodes presumably functional genes for type I, II, III, IV, V, and VI (T1-6SS) secretion systems, many of which are located on GI. The T3SS and T4SS both secrete effector molecules into host cells.[47,48] T2SS, T3SS, and T4SS also appear to contribute to the establishment of mutualistic, intracellular invertebrate-bacteria associations.[26] Many of these secretion systems (T2,3,4, and 6SS) are also found in the family-related bacterium, *Hahella chejuensis* KCTC 2396^T,[16] and *E. elysicola* DSM 22380^T (T2,3SS), which suggests that at least the T2SS and T3SS may have originated prior to the octocoral symbiotic association.

EF212^T has also genes encoding eukaryotic-like ankyrin repeats. Most of these eukaryotic-like proteins are located adjacent to mobile elements, suggesting that they may be secreted into the host cytoplasm to manipulate molecular processes in host cells.[47]

Finally, there is a phosphatidylinositol signaling system present, which may be involved in octocoral larvae metamorphosis.[25] Also present are several quorum-sensing, chemotaxis, motility, and biofilm formation genes (LuxR family response regulators, AI-2 TqsA, Rsb, MalE, CheY, Fil, Mot, Pil, and Spo0), which may further interact with the holobiont system.[60]

5.4.6 Primary vs. Secondary Endosymbionts of E. fusca

In addition to the abundance and ubiquity of EF212^T in the *E. fusca* metagenome, there is also a stable presence of a *Mycoplasma* relative.[5] *Mycoplasma* are known to have extremely reduced genomes and are often found in association with eukaryotes as commensals or parasites.[49,50] In corals, they are hypothesized to be chemosynthetic commensalists that provide a source of nutrition for the corals either directly through the breakdown of hydrocarbons,[51,52] or indirectly, as the base of the food web.[53,54] This may suggest that the *Mycoplasma* relatives are

the "original," primary, obligate endosymbionts of *E. fusca*, while the *Endozoicomonas* are newly-established, secondary, facultative endosymbionts that may eliminate the *Mycoplasma* relative over time if their functions were to be replaced by the *Endozoicomonas* symbiont.[26]

5.5 Conclusions

From this cursory analysis of the genome of *Endozoicomonas euniceicola* EF212^T, it can be concluded that this bacterium is a recent symbiont of *Eunicea fusca* and likely provides nutrients to the holobiont that cannot be obtained from the surrounding, oligotrophic environment or biosynthesized by the octocoral or other associated microbes. In order to establish if EF212^T is a true symbiont of *Eunicea fusca*, fluorescence *in situ* hybridization, using specifically-designed primers, and transmission electron microscopy should be carried out to determine the location of this bacterium within the octocoral and to verify its role in nutritional provision.

There is no evidence of a fuscol biosynthetic gene cluster, but other secondary metabolite gene clusters common in many marine bacteria, including a siderophore and bacteriocin gene cluster, further support a nutrient provisional lifestyle (siderophore gene cluster) and may support previously observed antimicrobial activity through bacteriocin production.

A more comprehensive analysis of all genes in the EF212^T genome, as well as *Endozoicomonas gorgoniicola* PS125^T's genome will provide greater insights into this important and ubiquitous group of coral-associated microbes. Furthermore, as more genome sequencing data for *Endozoicomonas* spp. become available, comparisons can be made between the different phylotypes associated with specific species of corals and other marine invertebrates from diverse environments.

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CHAPTER 6: OVERALL CONCLUSIONS AND FUTURE PERSPECTIVES IN OCTOCORAL MICROBIAL DIVERSITY AND MARINE NATURAL PRODUCTS RESEARCH

6.1 Summary of PhD Thesis Objectives and Results

6.1.1 Chapter 2: Culture-Independent Study of Eunicea fusca and Related Plexauridae Octocorals to ***Determine "Total"*** *Microbial Composition at the Time of Collection*

Chapter 2 (pp 27-113) explored the culture-independent microbial diversity of *E. fusca* and related *Plexauridae* octocorals detailing their bacterial, fungal, dinoflagellate, and archaeal communities. Briefly, this study found two dominant groups of the bacterial community of *E. fusca*, most closely related to *Endozoicomonas* spp. and *Mycoplasma* spp. These bacterial counterparts were present in high abundances at different geographic locations and were spatially stable associates at all sites. Thus, these putative symbionts are likely involved in the health of the octocorals through nutritional provision or marine natural product (MNP) production. Furthermore, all octocorals contained the dinoflagellate *Symbiodinium* clade B1/B184. Future metagenomic studies, as well as culturing studies, are required to explore the biosynthetic potential and functional roles of the stable bacterial and dinoflagellate associates within the octocoral. This study also found that *E. fusca* had a different bacterial community than the other *Plexauridae* octocorals and from the surrounding seawater, which demonstrates species-specificity and the ability to attract and/or exclude certain microbial associates. In contrast to the bacterial and dinoflagellate communities, the fungal and archaeal communities did not show consistency within a geographic location or within coral host species, suggesting that specific species of fungi and archaea may not be essential for octocoral holobiont functioning. More comprehensive culture-independent studies are required to further understand these microbial associations.

6.1.2 Chapter 3: Culture-Dependent Study of E. fusca and Related Plexauridae Octocorals and the Search for Marine Natural Products from Cultured Microbes

Chapter 3 (pp 114-201) explored the culture-dependent microbial community of *E. fusca* and related *Plexauridae* octocorals. In addition to characterizing the culturable microbial communities of these octocorals, this study compared the cultured isolates to the culture-

independent community to determine if any of the cultures were dominant members of the culture-independent community. Finally, selected microbial cultures were further investigated for their antimicrobial activity and for fuscol production. Using a variety of culturing and isolation methods, such as particle-filtration[1] and diverse media, this study led to the isolation of 148 unique microbes (137 bacteria, 11 fungi), 31 (22.6%) of which were novel species of bacteria. Two of the novel species were *Endozoicomonas* species, *Endozoicomonas euniceicola* EF212^T spp. nov. and *Endozoicomonas gorgoniicola* PS125^T spp. nov. Having these bacteria in culture that had the same 16S rDNA sequence to dominant members of the culture-independent community provided the extraordinary opportunity to explore their ability to produce MNPs and examine their role(s) in the *E. fusca* holobiont. The *Endozoicomonas* spp. nov. MNP profiles were explored in a probiotic and fermentation study, but no MNPs were detected using these methods. However, fermentation studies of other associated bacteria led to the isolation of novel MNPs produced by a *Euzebyella* sp. (a 2,5-dialkylresorcinol with good Gram-positive bioactivity against MRSA, VRE, and *Staphylococcus warneri*) and a *Labrenzia* sp. (novel fatty-acid derivatives). No fuscol producer was identified under the fermentation conditions used in this study, but the extensive cultured library should be further explored under a greater variety of fermentation conditions in future studies to explore its full biotechnological potential.

6.1.3 Chapter 4: Formal Characterization of Endozoicomonas spp. nov.

Chapter 4 (pp 202-232) formally characterized the two novel *Endozoicomonas* species using a polyphasic approach. Briefly, this study revealed that the two *Endozoicomonas* spp. were novel species based on their genotype, chemotype, and phenotype relative to one another, as well as to the other characterized strains. The low DNA-DNA re-association values (4.8-34.8%) between members of this genus confirmed that these bacteria were novel species. This study also amended the genus description to include a wider variety of respiratory quinones and fatty acids, as well as expanded the G+C mol% content. As more members of this genus are isolated and formally characterized, it is likely that more amendments will be made to the genus, or, it may be

argued that the genus should be split into multiple genera based on the low genomic similarity of the phylotypes cultured from diverse marine invertebrates. The genome of these bacteria was further explored in Chapter 5 to predict potential roles of these symbiotic microbes within the octocorals and to explore their biosynthetic potential.

6.1.4 Chapter 5: Genome Sequencing of Endozoicomonas spp. nov. to Determine their Function in Plexauridae Octocorals and Other Marine-Invertebrates

Chapter 5 (pp 233-264) explored the genomic sequences of EF212^T and PS125^T. This study found that both *Endozoicomonas* spp. nov. have genomes greater than 6 megabase pairs (Mb), demonstrating the potential to house secondary metabolite gene clusters.[2] Annotation of the estimated 6.3 Mb EF212^T genome (incomplete genome composed of 19 scaffolds) revealed an abundance of environmental information processing and membrane transport genes, as well as many genes involved in carbohydrate, nucleic acid, amino acid, nitrogen, sulfur, and methane metabolism. Extracellular hydrolytic enzymes involved in the degradation of organic and inorganic substrates were also found. There were an abundance of mobile elements (*e.g.* transposons) associated with genomic islands (*i.e.* horizontally transferred genes) containing complete (bacteriocin and siderophore) and partial (antimicrobials and toxins) secondary metabolite gene clusters. Also present were all of the genes involved in the terpene deoxyxylulose pathway, but the presence of ubiquinone and menaquinone genes suggested that this terpene precursor biosynthetic pathway was likely involved in the biosynthesis of these isoprenoid quinones involved in aerobic and anaerobic respiration, respectively. No terpene synthases for fuscol biosynthesis were found. Also present were genes involved in the demethylation and cleavage of the dinoflagellate byproduct, dimethylsulfoniopropionate (DMSP).

From this cursory annotation analysis, it was predicted that EF212^T may provide *E. fusca* with nutrients that the octocoral itself cannot assimilate or obtain from the oligotrophic reef environment. Furthermore, certain characteristics of the genome (*e.g.* the discrepancy in the GC content between the coding (~48.7%) and non-coding (~44.1%) regions,[3] the wealth of

pseudogenes,[4,5] and the abundance of mobile elements and genome rearrangement[6]) suggested that EF212^T is in an early stage of symbiosis with *E. fusca*. More in-depth analyses of the EF212^T and PS125^T genomes will be carried out in future studies to further explore their roles in the *Plexauridae* octocorals.

6.2 Overall Conclusions and Future Directions from this PhD Research

From this PhD research, the culture-independent and –dependent studies demonstrated that the unexplored microbiomes of *E. fusca* and related *Plexauridae* octocorals provided distinct and diverse microbial communities. Having knowledge of the healthy microbial communities of these octocorals can be used in the future to determine the health status of these octocorals in the ever-changing ocean environment. Future studies should explore the microbial communities of additional samples of Caribbean *Plexauridae* octocorals over spatial and temporal scales in order to ascertain the critical members of the microbial communities, such as the *Endozoicomonas* spp. and *Mycoplasma* spp. relatives. Additionally, the genomes of the *Endozoicomonas* spp. cultured in this study should be published in order to fill in the gap of knowledge about the roles these bacteria play in nutrient cycling in octocorals and other marine invertebrates.

The diverse culturing and isolation techniques employed in the culture-dependent study permitted the isolation of unique and novel microbes. The MNPs analysis suggested that some of these cultured microbes possessed chemically-diverse metabolisms, which ultimately led to the isolation of novel, microbial MNPs. In particular, octocoral microbes should be explored in the future as a source of novel antimicrobials, as this study and many others[8-11] have shown that coral-associated microbes possess antimicrobial activity. The isolation of novel antimicrobials with new mechanisms of action may one day lead to life-saving medicines treating infectious diseases that are becoming increasingly resistant to available treatments. Additionally, even though no fuscol producer or diterpene biosynthetic pathway was discovered in this study, future studies should further explore *E. fusca* and its microbial community to ascertain the true biosynthetic source of these desired MNPs.

6.3 Future Perspectives in Octocoral Microbial Diversity and Marine Natural Products Research

Unique marine microenvironments, such as octocorals, abound with unique and novel microbes that are potential sources of novel MNPs. As well, they may provide a sustainable supply of MNPs originally isolated from the octocoral hosts. Thus, uncovering octocoral microbial diversity through culture-independent and –dependent methods is essential for us to discover novel microbial MNPs and/or novel microbial sources for known MNPs. Additionally, genome sequencing of stable, culturable octocoral microbial associates (*e.g. Endozoicomonas* spp.) and/or the isolation of MNP gene clusters from stable, yet uncultivable octocoral associates (*e.g. Mycoplasma*-relatives) in a complex metagenomic mixture will allow us to further explore the biosynthetic pathways of octocoral-associated microbes. These methods will answer fundamental questions about the true biosynthetic source of many known MNPs (possibly providing solutions to MNP supply issues) and allow for the discovery of novel MNPs encoded in the genomes, as well.

As these 'omic' techniques advance, new bioinformatics approaches will be needed to dissect all of the generated data. Additionally, even though MNPs may be uncovered using these methods, there is still a need to identify their mode of action and carry out structure-activity-relationship studies to develop MNPs into pharmaceutical drugs. Thus, the future of MNP drug development will be quite interdisciplinary requiring scientists from diverse backgrounds (*e.g.* organic chemistry, synthetic chemistry, medicinal chemistry, molecular biology, microbiology, metagenomics, genomics, bioinformatics, and many more) all working together to develop promising MNPs as lead drug candidates.

Due to their molecular complexities and unique mechanisms of action, MNPs will continue to make up a very significant portion of pharmaceutical drugs and therapeutic agents in the years to come. To date, seven MNPs have entered the pharmaceutical market as approved drugs,[7] and the full pipeline of MNPs in clinical and preclinical trials suggests that the marine

environment provides an excellent source of agents for drug discovery. As Cragg and Newman eloquently said, "Mother Nature has had three billion years to develop and refine her chemistry,"[7] and we are only now scratching the surface of the ocean's depths of biological and chemical diversity.

6.4 References

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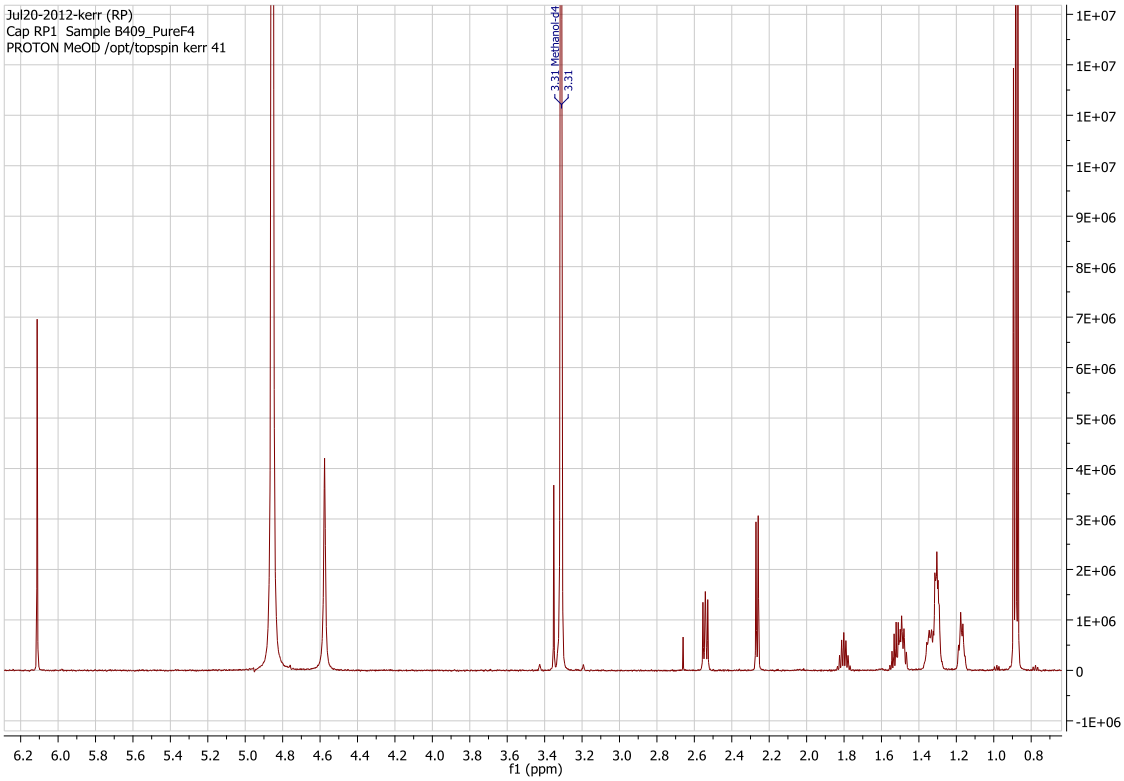
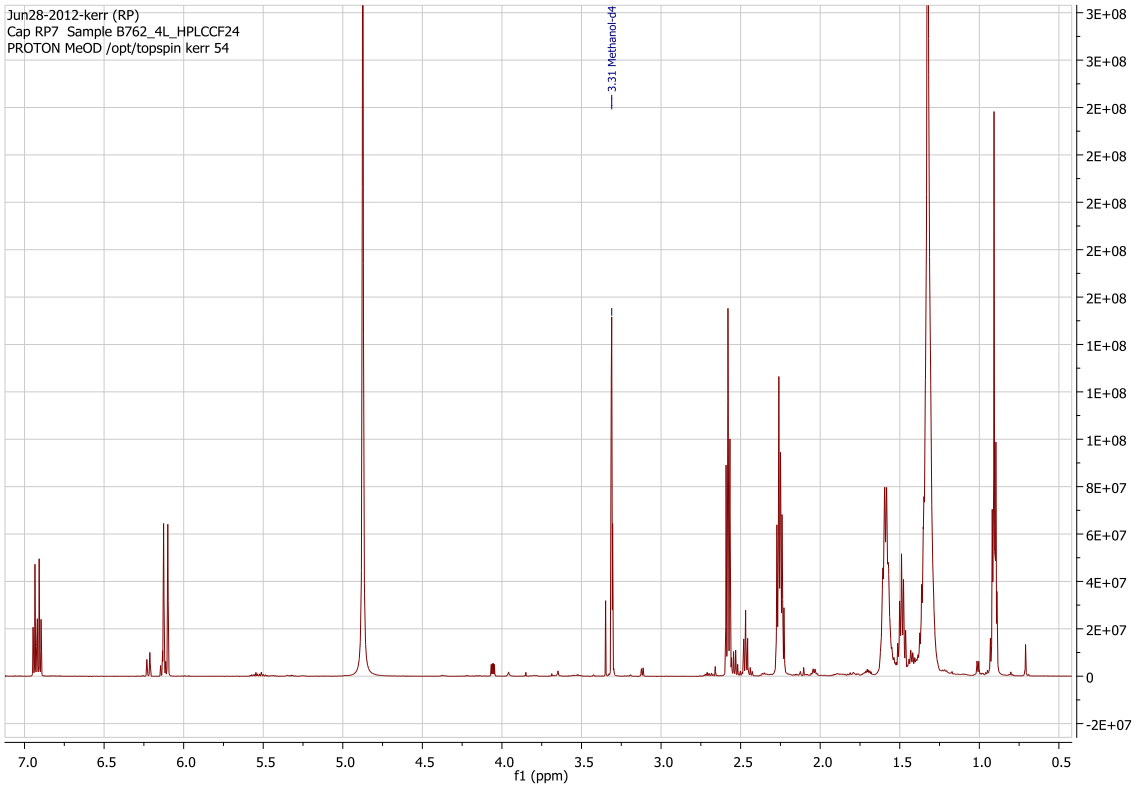
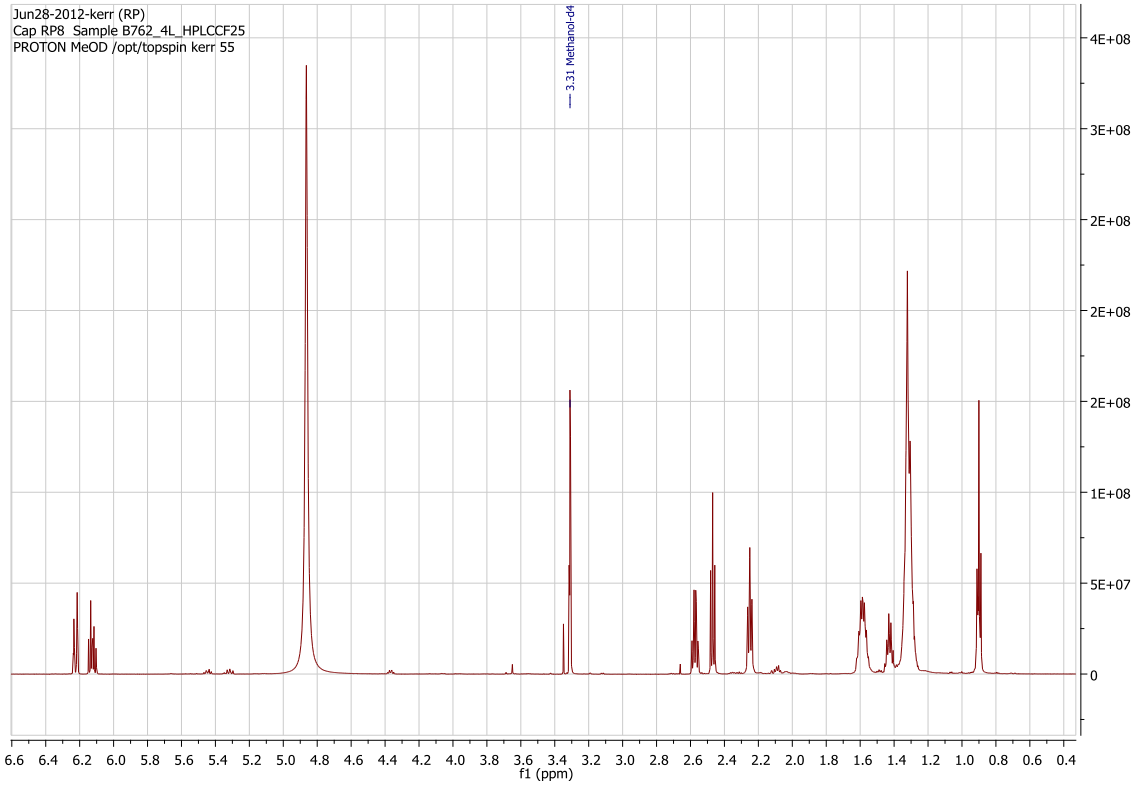
APPENDIX A: ^{1}H NMR SPECTRA FOR NOVEL COMPOUNDS (CHAPTER 3)



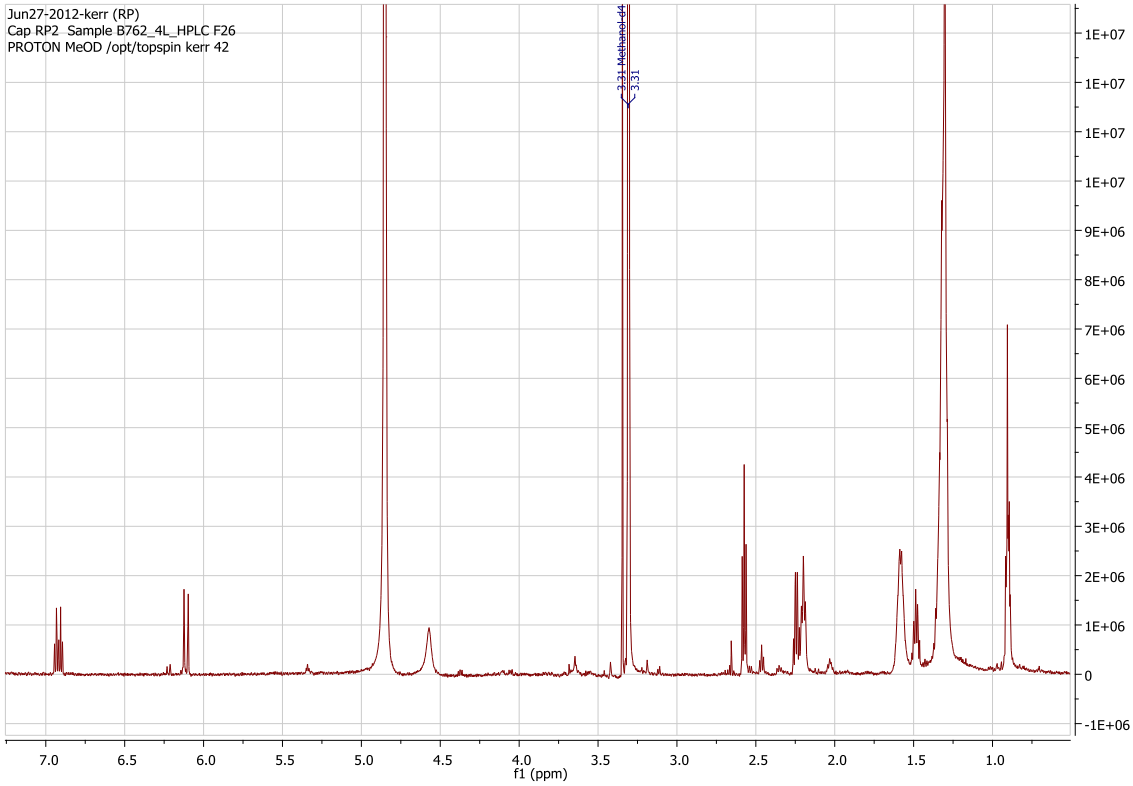
Supplementary Figure 3.1 ^{1}H NMR of novel 2-isononyl-5-isobutylresorcinol isolated from *Euzebyella* sp. EF1C-B409 (Chapter 3, Figure 3.14, p. 162).



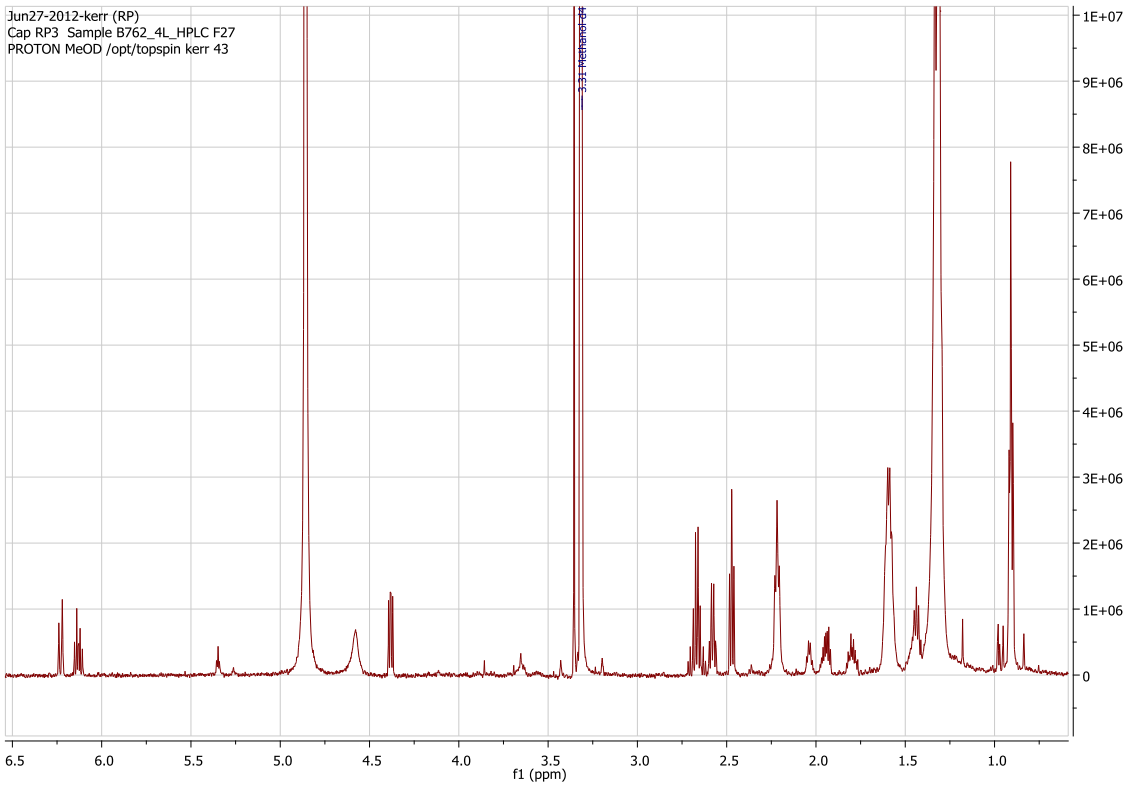
Supplementary Figure 3.2 ^{1}H NMR of novel (*E*)-10-oxooctadec-11-enoic acid isolated from *Labrenzia* sp. EF3B-B762 (Chapter 3, Figure 3.18, p. 168).



Supplementary Figure 3.3 ^{1}H NMR of novel (*Z*)-10-oxooctadec-11-enoic acid isolated from *Labrenzia* sp. EF3B-B762 (Chapter 3, Figure 3.19, p. 169).



Supplementary Figure 3.4 ^{1}H NMR of predicted (*E*)-12-oxoicos-13-enoic acid isolated from *Labrenzia* sp. EF3B-B762 (Chapter 3, Figure 3.23.A, p. 175).



Supplementary Figure 3.5 ^{1}H NMR of predicted (*Z*)-12-oxoicos-13-enoic acid isolated from *Labrenzia* sp. EF3B-B762 (Chapter 3, Figure 3.23.B, p. 175).